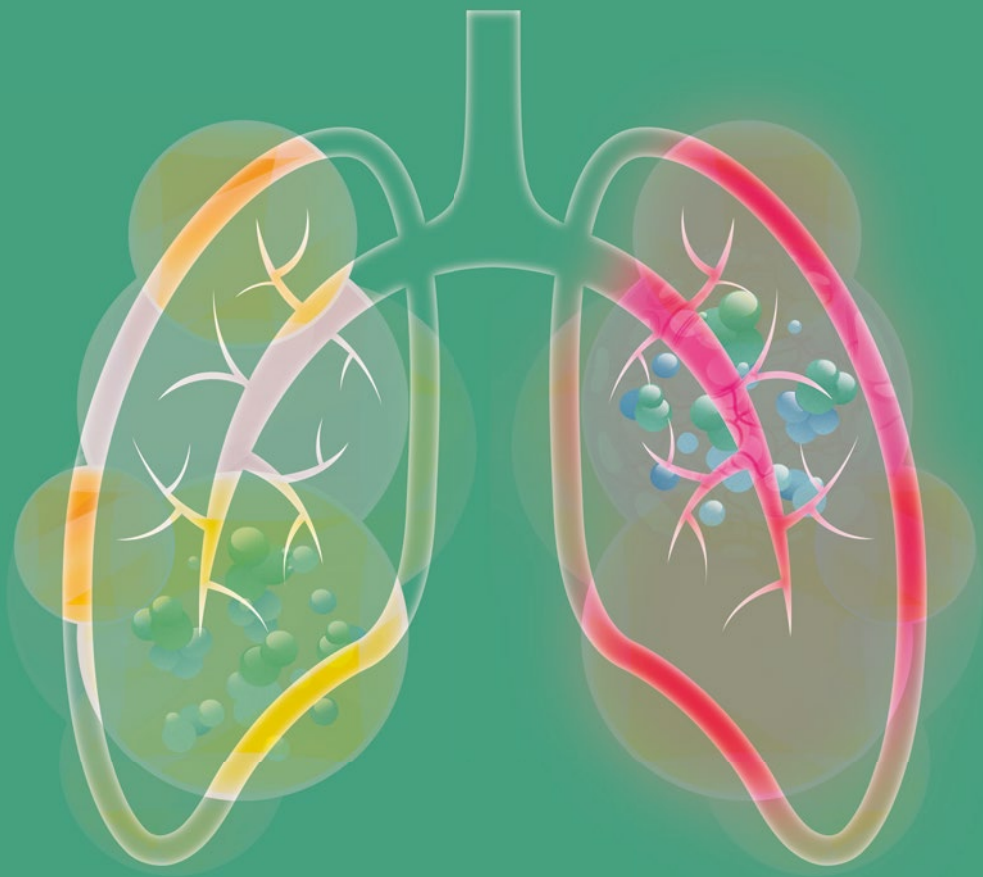
Determinants of disease severity in children with viral lower respiratory tract infections



Inge Ahout

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Thesis Radboud university medical center of the Radboud University Nijmegen, The Netherlands, with summary in Dutch.

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1

General introduction and outline of the thesis

Introduction

Acute lower respiratory tract infections (LRTI), including pneumonia and bronchiolitis, are the leading cause of childhood hospitalisation and mortality in children between two months and five years of age.[1-3] The burden of LRTI is high especially in developing countries. One of the millennium goals was to reduce under-five mortality by raising political awareness, implementation of health care programs, expanding vaccine coverage, improve healthcare systems, and focus on sociodemograpic factors.[1,4] These, and other actions resulted in more than 50% reduction in deaths between 1990 and 2015. Despite this achievement, it has however been estimated that LRTI still causes 1.4-2 million deaths each year and remains the major reason for childhood hospitalization.[1,5] Well known bacterial pathogens causing LRTI in children include *Streptococcus pneumoniae*, *Haemophilus influenzae, Staphylococcus aureus* and *Bordetella pertussis.* The incidence of bacterial LRTI has substantially decreased as a result of vaccination programs. However, studies using multiplex PCR techniques show that most cases of LRTI are caused by viruses, especially in children under five years of age. Jain *et al.* reported that 1472 (66%) of 2222 children with a radiologically confirmed community acquired pneumonia had a viral infection, whereas only 175 (8%) of the children had a confirmed bacterial infection.[6] Similar findings were recently published by Berg *et al.* in a cohort of patients with a high coverage of pneumococcal vaccination.[7] The most commonly detected virus is respiratory syncytial virus (RSV) followed by rhinovirus, human metapneumovirus, parainfluenza virus and influenza virus. The two recent studies of Jain *et al.* and Berg *et al.* highlight the importance of viral LRTI in children and the need for a better understanding of the pathophysiology of these infections in order to develop improved treatment strategies. This thesis presents epidemiological, diagnostic, clinical and laboratory (translational) studies on childhood infections by two major respiratory viruses: RSV and influenza virus.

Influenza virus

Influenza virus can cause severe respiratory disease in young children under 5 years of age. Influenza increases the risk of hospitalization in this age group (<5 years of age) 12-fold compared to the risk in children aged 5-17 years. [8] Although respiratory symptoms form the most important clinical presentation of influenza infections, influenza is also associated with muscle pain, malaise and neurological symptoms. Additionally, gastro-intestinal symptoms are often seen in children.[9,10] The exact burden of disease caused by influenza is difficult to estimate, since influenza causes a broad range of clinical symptoms and seasonal outbreaks overlap with those of other respiratory viruses such as RSV. In addition, viral testing is not routinely performed. [9,11,12] Most children have encountered influenza A virus before the age of 6 years. [13,14] Average hospitalization rates in children are 0.4-1.5/100,000 (95% CI 0.2-1.9). However, in

children younger than 6 months the hospitalization rate can be as high as 100/100,000, whereas emergency room visits vary from 6-27 per 1000 children (95% CI 4-33).[9] Healthcare costs, morbidity and mortality caused by influenza can be reduced by vaccination. Circulating influenza strains change rapidly during replication, which is called 'antigenic drift'. When adaptations in the hemagglutinin gene accumulate, the virus is no longer recognised by the human immune system.[15] This allows the virus to evade the antibody response raised by vaccinations and by previous exposure to influenza leading to re-infection. Every year, minor changes in the hemagglutinin and neuraminidase genes of influenza virus are analysed to determine the vaccine composition of the following year. When the seasonal prediction of the genetic makeup of the virus is correct, vaccines are effective and protective.[16-19] Three different vaccine types are currently available for children: a live attenuated influenza vaccine (LAIV) and a trivalent or quadrivalent inactivated influenza vaccine (TIV or QIV). Unfortunately, there are no vaccines available for children under 6 months of age, the age group with the highest risk for severe disease. There is an ongoing discussion on the vaccination strategy against influenza in children. Most European countries, including The Netherlands, use a risk group based approach.[20] However, risk groups are not well defined and data are often extrapolated from adult studies and not confirmed in studies in children. Moreover, a substantial proportion of hospitalized infants with influenza was previously healthy.[21] Currently a growing number of countries switched to vaccination of all (healthy) children to protect the population as a whole via herd immunity, and decrease the burden of disease on the healthcare system.[22,23] In the USA, Canada and England the ACIP, NACI and JCVI advise to vaccinate all children.[24-26] Finland, Austria, Switzerland, Malta, Slovenia, Slovakia, Poland, Estonia and Latvia recommend seasonal influenza vaccination for healthy children, although they differ in their selection of age categories.[20] In countries which recommend vaccination, the coverage among both healthy children and patients with an underlying condition remains low. For instance in the US 85% of the population is advised to be vaccinated. However, in practice only 50% of high risk patients is vaccinated and 27-45% of all children is completely vaccinated.[27,28] In the Netherlands only risk groups are vaccinated with a vaccine uptake between 52-70% in the at risk population.[20]

Respiratory Syncytial virus

Respiratory Syncytial virus (RSV) is the most frequently detected virus in children with LRTI [29] and in those requiring hospitalisation.[30] Infection usually starts as a common cold, but approximately 1/3 of patients will develop a lower respiratory tract infection with difficulties in breathing (nasal flaring, thoracic retractions, tachypnea, hypoxia) and feeding problems in the youngest.[31] By the age of 1 year 65% of infants have been infected with RSV of which 2-3% needs hospitalisation.[32] At the age of 2 years virtually all children have at least encountered RSV once.[33] Annual hospitalisation rates are

25-40 per 1000 infants in children under 1 year of age and 1.8 per 1000 children aged 1-5 year.[34] The course of disease of RSV in young infants is unpredictable. Patients can quickly deteriorate from mildly ill to becoming respiratory insufficient. This leads to frequent hospitalisation in very young infants for observational purposes. Risk factors for severe disease can be classified as virus specific, environmental and host specific.[31] RSV has adapted to the human host and evades and alters the immune responses resulting in an insufficient induction of long lasting immunity. In a recent meta-analysis Shi *et al.* confirmed the presence of previously well known environmental and host specific risk factors for the development of severe disease such as prematurity, low birth weight, siblings, maternal smoking, history of atopy, no breastfeeding, crowding and being male.[35] Age is considered the most important risk factor. This is a consequence of a more immature immune system, smaller airways and less energy levels combined with less effective coughing in young children.[36] Host specific risk factors include chronic diseases such as congenital heart or lung disease, which diminish an adequate broad immune response to RSV infection. Patients with immunodeficiency defects in either innate or adaptive immunity also have an increased risk for (severe) infection.[36] However, more than half of the patients admitted to the ICU with severe RSV disease was previously healthy.[31,37] Several investigators have tried to develop clinical prediction rules (CPRs) for children with RSV infections or bronchiolitis. These CPRs have different outcomes such as safe discharge from the emergency room, need for hospital admission or the prediction of the need for ICU admission. More than a hundred clinical prediction rules for children have been published but only few are used in clinical practice.[38] Maquire *et al.* and Ferrero *et al.* discuss the reasons why CPRs for children are not used in daily practise. First of all, many prediction rules use subjective criteria resulting in a large inter-observer variability. Validation of prediction rules in children is more difficult than in adults due to the relatively small numbers of affected children and rare outcomes. Moreover, many clinicians seem to expect a 100% outcome, whilst CPRs are only used as a tool to improve clinical decision making and increase the sensitivity of clinical judgement.[38,39] Validated CPRs are available for children with meningitis and pneumonia. These decrease the use of unnecessary antibiotics with 52 and 55.6%, respectively. [40-43] Validation of CPRs for RSV is often missing, or appears problematic due to subjective criteria.[44,45]

To date, it is not known how immune responses against RSV develop in time and where in this process the course of disease is being determined. However, the early innate immune response is thought to play a major role in the pathogenesis.[36,46] Severe bronchiolitis is associated with airway obstruction caused by sloughing and apoptosis of airway epithelium, oedema of bronchioli, mucus production and infiltration of the airways with neutrophils and lymphocytes.[47,48] RSV has cytopathic effects, but the immune response is also thought to play an important role in the pathogenesis.[46,48] It remains a challenge to study the course of disease in these very young children, especially since the site of infection (the lungs) is difficult to sample. Alternatively, more research is necessary to study the inflammatory response at the level of the nasal

mucosa. The nasal mucosa forms the initial site of infection, and may therefore also be a factor to influence disease severity. In the last decade new techniques emerged to study the host response during infection. These allow to make a differentiation between viral and bacterial infections by a characterization of the transcriptional (RNA) host response against these pathogens.[49-51] This response appears to be so specific that it even allows identification of the individual pathogen causing the disease. Mejias *et al*. successfully explored the use of host transcriptome analysis to develop a scoring system for severity of disease called the molecular distance to health.[52]
We conducted and describe in this thesis an observational cohort study, called Venturius, within the Virgo-consortium (www.virgo.nl). In this study we compared patients with mild disease (no need for supplemental oxygen) admitted for observation or sent home after emergency department visit with patients admitted to the hospital with moderate disease (need for supplemental oxygen) and with severe disease (need for mechanical ventilation on the ICU). The study design is depicted in figure 1.

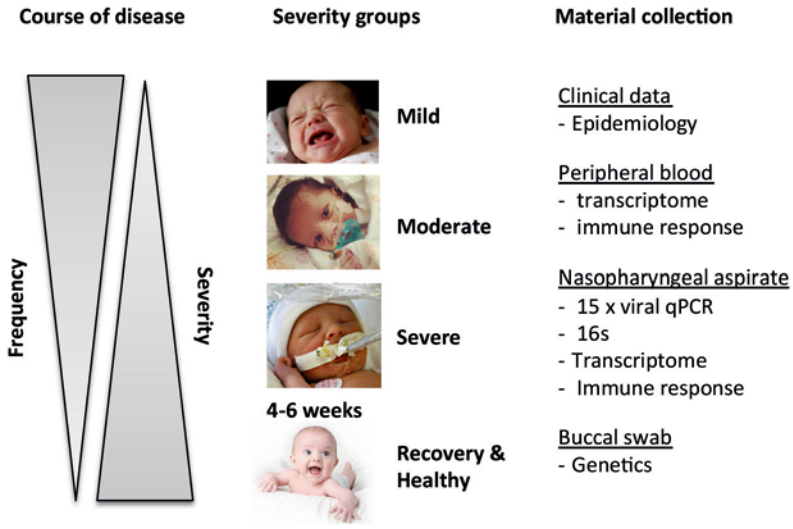

Figure 1.1 Study design Venturius study. Patients presenting at the emergency room from one of the two hospitals in Nijmegen with signs and symptoms of a viral RTI were asked to participate in this study. After parental consent was obtained samples (6 ml blood, nasopharyngeal aspirate and saliva) were collected within 24 hours after presentation in the hospital. The children were retrospectively allocated to one of three severity groups. Patients admitted to the ICU from the Radboud university medical center in Nijmegen or the Sophia Children's Hospital in Rotterdam were also asked to participate and were included within 24 hours after admission. Recovery samples were taken after 4-6 weeks. A healthy control group consisted of healthy infants who needed surgery.

Aim of this thesis

The aim of this thesis is to identify risk factors and immunological determinants that are important in the development of severe viral respiratory tract infections in young infants.

Objectives of this thesis

- To identify risk groups for a severe course of influenza infection in children.
- To discover and study biomarkers that reflect the course of disease and provide insight in the pathophysiology of RSV infections.
- To explore factors at the nasal mucosa that influence the immune response against RSV.

Outline of the thesis

In the **first part** of this thesis we focus on the emergence of pandemic H1N1 **influenza virus**. Many new viruses have been discovered in clinical specimens since the introduction of molecular techniques to identify nucleic acid sequences of viral DNA or RNA. In **Chapter 2** the clinical features of novel respiratory viruses including the H1N1 virus, which emerged during the last decade are described. pH1N1 caused a major outbreak in The Netherlands In the respiratory season of 2009-2010. A description of the first children hospitalized with pH1N1 in two Dutch hospitals is presented in **Chapter 3**. During this pandemic, influenza vaccination was recommended for all children and pregnant women. This resulted in a public debate on the benefits of influenza vaccination and the scientific, economical and ethical grounds on which this decision was made. In **Chapter 4** we studied the course of disease of influenza infection in a nationwide retrospective study on the clinical characteristics of hospitalized children. In this study we focused on the risk factors of severe disease. In **Chapter 5** the arguments in favor of routine influenza vaccination for children are weighed against the criteria to introduce a new vaccine into the Dutch national immunization program.

The **second part** of this thesis describes studies on infections by the **respiratory syncytial virus**. Because the course of disease is difficult to predict in young infants with a RSV infection, there is a need to detect novel plasma or mucosal markers for severe disease. In **Chapters 6** and **7** inflammatory proteins were measured in the plasma of children with RSV infection and levels were correlated with disease severity. In severe bacterial infections and sepsis, the expression of surface markers on circulating leukocytes is correlated with disease severity and immune paralysis. These markers have not yet been tested in children with RSV infections. In **Chapter 8** we studied the expression of surface markers on circulating monocytes of patients with non severe or severe RSV infections and compared the data with those from cells derived from recovered and healthy infants. Furthermore, we tested the effect of LPS on monocytes of these patients to establish the presence of immunoparalysis. Surface marker expression and protein production are partially regulated at the transcriptional

level. We therefore performed in **Chapter 9** micro-array transcriptome studies on peripheral blood mononuclear cells of children with infections by RSV. In these experiments we wanted to find biomarkers for a severe course of disease. In **Chapter 10** we describe a transcriptome analysis performed on whole blood and used the data to further optimize the statistical methods in order to detect a combination of biomarkers to predict disease severity. In **Chapter 11** a different analysis was used on the same data, based on modules associated with specific cell-type activation profiles. We intended to identify cell subset populations involved in the pathogenesis of severe RSV disease. In addition we used 'distance to health' measurements to predict disease severity.

The **last part** of this thesis focuses on studies of nasal mucosal samples. The nasal mucosa is the primary site of initial RSV infection. We hypothesized that crucial steps in the development of and susceptibility for severe disease are initiated at the mucosal level. We first studied the host response by studying gene expression in cells derived from nasopharyngeal washes during infection. In addition, we investigated the composition of bacteria residing in the nasal cavity (microbiome) the level of maternal antibodies. In **Chapter 12** we describe transcriptional markers, that reflect the level of disease severity. Presence and quantity of frequent colonizers of the respiratory mucosa was determined in the nasopharynx of children with and without RSV infection and correlated with inflammatory parameters and disease severity, as described in **Chapter 13**. Immunoglobulin levels present at the nasal mucosa were measured and these levels were correlated with the viral load and inflammatory proteins in the nose (**Chapter 14**). Finally, in **Chapter 15** all studies are summarized, discussed in broader perspective and new ideas for future studies are proposed.

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Part I

Influenza virus

2

Elucidation and clinical role of emerging viral respiratory tract Infections in children

Inge M.L. Ahout, Gerben Ferwerda and Ronald de Groot

Hot Topics in Infection and Immunity in Children IX,
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Abstract

Acute respiratory viral infections (ARVI's) are the most common infectious disease in humans. With the appearance of molecular techniques the recovery of viruses has dramatically increased. Nowadays virologists can quickly discriminate virological families and related viruses from emerging viruses and consequently identify novel viruses. Many new respiratory viruses have been identified in children in the past 15 years. In this review we shortly discuss novel respiratory viruses and their pathogenic role in pediatric respiratory disease. Advantages and drawbacks of the technique and our current knowledge will be discussed. We will conclude this review with a general discussion on the future role of molecular diagnostic virology in the clinic.

Introduction

General (Respiratory Viruses in Pediatrics)

Acute respiratory viral infections (ARVI's) are the most common infectious disease in humans. They occur more frequently in children than in adults (6.1 episodes per year under the age of 1, 3–6 episodes per year between the age of 1–5 and 2.4 episodes per year between the age of 15–19). Disease severity depends on age, underlying condition and type of virus. ARVIs account for huge numbers of doctor's visits and days lost from work and school. They are a leading cause of global mortality and morbidity in children. Moreover, respiratory viral infections are an important driver of unnecessary usage of antibiotics. Unfortunately prevention and treatment of the majority of respiratory virus infections is not possible with the exception of influenza.[1]

Although much research has been done on the epidemiology and burden of viral respiratory tract infections the size of the problem is underestimated. Due to the lack of routine testing for (multiple) viruses and the limitation that a majority of infected patients will not visit a doctor.

General Introduction Molecular Techniques (Discovery Novel Viruses)

With the appearance of molecular techniques the recovery of viruses has dramatically increased. Before their use approximately 50–80% of the viral tests remained negative.[2] Due to the introduction of PCR and the discovery of novel viruses this proportion decreased to 3–15 %. However, such recovery rates are largely dependent on the selection of the patient group.[3-5] The combination of high sensitivity, multiplex options and quantification was essential for some of the new insights in viral epidemiology. This could not have been achieved with conventional viral diagnostics such as culture and immunefluorescence assays (FDA).

The identification of respiratory viruses in a clinical context can also guide diagnostic and treatment strategies. Bonner *et al.* revealed that a known viral aetiology of disease results in decreased use of additional tests such as X-rays or blood examination, shorter hospital admission and less frequent use of antibiotics.[6] However, molecular diagnostics have also created new dilemmas. For example, the identification of respiratory viruses in asymptomatic children, the occurrence of many viral co-infections, concerns about the pathogenic capacity of certain viruses and the value of quantitative measurements.

General Introduction on Emerging Viral Diseases

Some of the advantages of the new genetic (e.g. sequencing) and molecular techniques became clear during outbreaks of novel emerging viruses. Emerging viruses can be classified as (1) previously unknown viruses or (2) previously known viruses that have significantly increased in incidence.[7] Nowadays virologists can quickly discriminate virological families and related viruses from emerging viruses and consequently identify novel viruses.

Table 2.1 Emerging viruses from the last 2 decades

Family	Virus	Year of discovery
Hendra-/NipahV	Paramyxovirus	1995
AIV's	Influenza virus	1997
hMPV	Paramyxovirus	2001
SARS-CoV	Coronavirus	2003
HCoV-NL63	Coronavirus	2004
HCoV-HKU1	Coronavirus	2005
HBoV	Parvovirus	2005
HPeV4	Parechovirus	2006
HPeV5	Parechovirus	2006
HPeV6	Parechovirus	2007
KIV/WUV	Polyomavirus	2007
H1N1V	Influenza virus	2009

The introduction of molecular diagnostics in medical virology has led to the identification of many new respiratory viruses in children in the past 15 years (**Table 2.1**). However, the pathogenicity of these viruses is not always clear and the clinical relevance is often poorly understood. Fredricks and Relman proposed seven rules which are necessary to demonstrate the causative relationship between a virus and disease. These rules are based on Koch's postulates and were adapted for nucleic acid based detection methods, location of the pathogen and quantification (**Table 2.2**).[8,9] These rules can help to interpret research on the role of novel respiratory viruses in disease and guide future research. It should also be stated that the clinical relevance is in some cases apparent, without extensive research to fulfil all requirements.
In this review we briefly discuss novel respiratory viruses and their pathogenic role in pediatric respiratory disease. We will conclude this review with general discussion on the future role of molecular diagnostic virology in the clinic.

Henipavirus (1994–1998)

Hendra Virus

The Hendra virus was first detected in a disease outbreak in 1994. It initially presented with a new respiratory disease in horses that was transmitted to two persons one of them died.[10] The virus belongs to the genus of *Henipavirus* within the family of the *Paramyxoviridae* family. It was initially named morbillivirus and later renamed Hendra virus (HeV) after the suburb where the outbreak occurred.[11,12] The virus itself is not very contagious. It spreads through direct contact between horses or during intensive contact between humans and severely ill horses. The animal reservoir appears to be the Flying-fox, in this population the Hendra infection is largely subclinical. The breading

Table 2.2 Koch's postulates adapted for viral infections [8,9]

A nucleic acid sequence belonging to a putative pathogen should be present in most cases of an infectious disease. Microbial nucleic acids should be found preferentially in those organs or gross anatomic sites known to be diseased, and not in those organs that lack pathology
Fewer, or no, copy numbers of pathogen-associated nucleic acid sequences should occur in hosts or tissues without disease
With resolution of disease, the copy number of pathogen-associated nucleic acid sequences should decrease or become undetectable. With clinical relapse, the opposite should occur
When sequence detection predates disease, or sequence copy number correlates with severity of disease or pathology, the sequence-disease association is more likely to be a causal relationship
The nature of the microorganism inferred from the available sequence should be consistent with the known biological characteristics of that group of organisms
Tissue-sequence correlates should be sought at the cellular level: efforts should be made to demonstrate specific in situ hybridization of microbial sequence to areas of tissue pathology and to visible microorganisms or to areas where microorganisms are presumed to be located
These sequence-based forms of evidence for microbial causation should be reproducible

season of Flying-foxes is a risk period for spread and the human risk group is defined as people with close and intensive contact with horses. Up till now fourteen outbreaks have been reported. [13] In five of these outbreaks humans were involved, resulting in five deaths. So far, only two persons survived an infection.
The case fatality rate (CFR) is over 50 %.[13] HeV in humans causes, after an incubation period of 5–21 days, a severe influenza like disease (fever, myalgia and headache) which can progress to pneumonia, respiratory failure and death.[10,14] An infection can also result in encephalitis with headache, fever and drowsiness. The encephalitis can occur after initial recovery from the illness. The Hendra virus genome is readily detected in several materials, e.g. blood, urine, nasal- and oropharyngeal swabs by RT-PCR. Next to this standard detection method the virus can be cultured in several cell lines, where it forms syncytia upon infection. ELISA serological tests are used for screening, however their diagnostic sensitivity is not yet established. Immunofluorescense assays and serum neutralization methods can also be used.[13,15]

Nipah Virus

In 1998 and 1999 two large outbreaks of respiratory disease in pigs and humans occurred. In Malaysia and Singapore 106 people died.[16] The causative agent had large similarities to the HeV and is the second member of the genus of *Henipavirus* within the *Paramyxoviridae* family. It was named Nipah virus (NiV) after the location of the first

human case.[17] This virus had already caused respiratory disease in pigs until late 1996. Like HeV it shares the bat as a natural reservoir. NiV virus is very contagious among pigs and spreads through the respiratory route or directly by the transport of infected pigs. Initially humans became infected via direct contact with pigs, although food borne transmissions were also reported. Initially the case fatality rate was 38.5 %.[14,18] Since the initial outbreak almost yearly new outbreaks emerged in Bangladesh and India causing fatal encephalitis in humans. Notably, the CFR's of these outbreaks increased to 92 %. Also the transmission changed: starting from pigs, soon cows could transmit the virus. Later human to human and nosocomial transmission was demonstrated.[19-21] It has been hypothesized that there were multiple introductions of viruses in the human population, explaining the unique genetic signature of isolated viruses nowadays.[18] These genetic differences may be the reason for the increase in CFR and differences in clinical manifestations and transmission. The clinical manifestation of a Nipah infection differed per outbreak. Incubation periods differ from an average of 2 weeks in Malaysia to 1 week in Bangladesh. The infection can be asymptomatic, but often starts with influenza-like symptoms of fever, headache, myalgia, vomiting and sore throat. Patients can recover or develop signs of encephalitis or sometimes atypical pneumonia or acute respiratory distress. In severe cases the encephalitis includes the brain stem or progresses to a coma within 24–48 h.[18,19,22] Around 20% of the cases are left with residual neurological symptoms, including personality changes. In comparison with outbreaks in Malaysia and Singapore the Bangladesh and Indian patients experienced more profound respiratory symptoms with case rates of 14, 27, 70 and 51 %, respectively.[19] A Nipah infection can be diagnosed in serum urine and cerebrospinal fluid (CSF) by RT-PCR.[23] Also culture in cell-lines, ELISA for anti-HeV IgG and IgM in serum and CSF, serum neutralization assays or immunofluorescence assays are used.[18,19,24,25]

Patients with both Hendra and Nipah virus infections are treated supportive, antiviral therapy is not effective.[22] Prevention is based on careful hygiene, quarantine and safe disposal of animal carcasses.[13,14] Currently, there are no vaccines available. However, several therapeutic agents seem effective *in vitro* and in some animal models .[18]

Avian Influenza Virus (1997)

The first cases of avian influenza virus (AIV) infection were reported in 1997 in Honk-Kong.[26] This influenza A (H5H1) originated completely from strains circulating in wild birds and poultry.[27] The avian influenza virus undergoes rapid genetic and antigenic evolution reflected by the occurrence of different clades with distinct phenotypes.[28,29] The majority of human cases had direct contact with poultry or could be related to outbreaks in wild birds.[20] There is limited transmission from human to human, although some epidemiological studies suggest it is possible.[30] The median age of patients is around 18 years and the mortality rate is extremely high between the age of 10 and 19 years (61 %). Yearly H5N1 outbreaks in humans have been reported in Asia, Africa and

Eurasia.[31] These epidemics are all related to outbreaks of avian influenza in wild birds or poultry during the colder seasons.[32,33] The incubation period of H5N1 is estimated to be 2–7 days.[34] The disease typically manifests as a severe pneumonia which often progresses to respiratory failure and death within 10 days (case fatality rate up to 90 % in children). It appears that in children cases may occur without pneumonia. Detection of viral RNA by (RT-) PCR is the best method for the diagnosis of H5N1, preferably using throat swabs.[35] Because of genetic variably of the virus, primers need to up-dated frequently. The available immune-assays for detection of H5N1 are not sensitive enough for clinical purposes and cannot differentiate between human and avian subtypes of influenza A. Seroconversion after 2–3 weeks can be used to confirm H5N1 infection and can be used for epidemiological studies.[35] Early treatment with oseltamivir is recommended based on some evidence that it increase survival rates.[36] There are differences in susceptibility to oseltamivir between the different clades of H5N1 circulating in different parts of the world. Combination of oseltamivir with amantidine can be given if the circulating H5N1 is susceptible to both agents. Currently, it is possible to produce vaccines that inactivate H5 influenza A strains. However due to the circulation of different clades and the rapidly changing antigenicity of H5N1 the need for the development of a new vaccine remains.[34,37]

Metapneumovirus (2001)

The human metapneumovirus (hMPV) was first discovered in the Netherlands in 2001 from a databank of samples from children with respiratory tract infections.[38] hMPV belongs to the genus *metapneumovirus* within the family of *Paramyxoviridae*. It is related to respiratory syncytial virus, both belonging to the *pneumoviridae* sub-family.
In both retrospective and prospective studies it has been shown that hMPV can be detected in 3.9–14.8 % of respiratory samples from children with respiratory disease.[39] This wide range reflects differences in the tested populations and the level of care. Co-infections with other viruses occur in 15–30 %.[40-42] hMPV is detected in up to 4 % of nasopharyngeal aspirates from healthy children, although percentages of less than 1 % are also frequently published.[43] Serological studies showed that all children by the age of 5 years had been in contact with the virus and that it has been circulating in the human population for over 50 years.[38] It has a seasonal occurrence with a peak incidence just after the influenza and RSV season.[43] Spread is thought to be via direct or close contact with respiratory secretions from an infected person with an incubation period of 3–5 days. Reinfections occur frequently in children, although symptoms are less severe.[44] Symptoms associated with hMPV infections are comparable with RSV (see **Table 2.3**) hMPV infections are, after RSV, the most frequent cause of bronchiolitis in young children and account for 5–15 % of all hospital admissions.[45] Hospitalization rates are highest among 6–12 month old children, remarkably older than for RSV.[46] There is an association between severe hMPV infection (bronchiolitis) and the development of wheezing in childhood.[47]

Table 2.3 Symptoms and diagnosis of hMPV mono infections in literature

Symptoms/diagnosis	Spread in literature (%)[a]
Fever[b]	36-80
Cough	67-99
Rhinitis	72-90
Wheezing	10-73
Respiratory failure	8
Oxygen 90%	32-85
Pharyngitis	24-66
Bronchitis	1-68
Bronchiolitis	11-51
Pneumonia	3-65
Otitis media	16

[a] Heikkinen *et al.* [121]; Aberle *et al.* [122]; Mullins *et al.* [123]; Chen *et al.* [124]; Manoha *et al.* [125]; Williams *et al.*[126]; Bosis *et al.* [127]
[b] Different definitions varying from > 37.5 to > 39 °C

hMPV can only be cultured in specific cell lines under specific conditions and is time consuming; therefore it has no role in a clinical setting. Real time PCR is the most sensitive test for hMPV detection in NPA and swabs and is therefore the common method in clinical and research settings.[48] RT-PCR also provides semi-quantitative information of the viral load (Ct value), which can be used to monitor treatment in a research setting.[49] Immunofluorescence assays are available for rapid detection of the virus in respiratory specimens; however, these tests are less sensitive than RT-PCR. Serology for hMPV has little additive value in the clinic because most children are seropositive in early childhood. Currently no vaccines against hMPV are available, though several candidates are being pursued.[45] Ribavirin, antiviral therapy, is effective *in vitro* against hMPV, though clinical data are sparce. Currently new therapies such as fusion inhibitors and siRNA's are being tested in murine models.[45]

Coronavirus (2003–2005)

Human corona viruses related to respiratory disease, 229E and OC43, have been known since the 1930s. They were recognized as the second most common cause of the common cold in humans.[50-52] A new strain of human coronavirus was identified in 2004 from a respiratory sample of a 7 month old infant with bronchiolitis and named NL63 (HCoV-NL63).[53] The HCoV-NL63 belongs to the genus Coronavirus within the family of Coronavidae. In retrospective cohort studies HCoV-NL63 have been identified in 1.7–9.3 % of respiratory samples from children with respiratory symptoms and occurs worldwide.[39] The virus is often found in combination with other respiratory viruses (57

%).[54,55] Peak incidence is found in the winter months and the incubation period is estimated 2–5 days.[56,57] HCoV-NL63 is associated with mild upper respiratory tract symptoms and less frequent with severe lower respiratory tract symptoms such as bronchiolitis.[58] Some studies have reported an association with croup.[55,59] HCoV-NL can be detected in respiratory specimens by RT-PCR which is the first choice for diagnosis. Immunoassays are available for rapid detection and distinction of different HCoV strains.[60] Different cell-lines are permissive for viral culture and used in a research setting. Currently no anti-viral treatment against HCo-ViNL63 is available, although several inhibiting compounds have been identified.[61]

A second novel human coronavirus was identified in 2005 in a 71-year-old man with pneumonia in China and named HKU1 after the Hong Kong University where it was found.[62] In a retrospective cohort studies the HCoV-HKU1 was identified in 1–3.1 % of respiratory samples in which no other virus was detected, from children with upper and lower respiratory symptoms with a higher incidence in children younger than 6 months.[39] The peak incidence of HCoV- HKU1 is in spring, early summer and winter with an incubation period of 2 days.[63] HCoV- HKU1 is mainly associated with upper respiratory tract symptoms in children and occasionally with pneumonia and bronchiolitis.[64] The first choice of assay for detection in respiratory specimens is RT-PCR. Coronaviruses exhibit substantial genetic variability hampering the development of pancorona primers and therefore specific primers for each strain have to be used.[65] There is no specific anti-viral therapy available against HCoV-HKU1.

Human Bocavirus (2005)

Human bocavirus (HBoV) belongs to the genus *Bocavirus* within the family *Parvoviridae* (and is closely related to the bovine parvovirus and canine minute virus). This virus was identified in 2005 by nucleic acid amplification (PCR) in respiratory tract specimens from Swedish children with lower respiratory tract infections.[66] In this study HBoV was detected in 3.1 % of hospitalized children below the age of three. Other studies detected HBoV in 3–19 % of children with respiratory symptoms depending on the sample type used (NPA and BAL *higher*, nasal swab *lower*) and the age of the patient (*higher* in younger children).[67,68] However, HBoV is frequently found in asymptomatic children (up to 40 %) or in combination with other viruses (up to 80%) in symptomatic children.[39] Based on these findings it is still unclear whether HBoV has a pathogenic role in respiratory disease. One study performed in a PICU suggests that the viral load (high titres) of HBoV may indicate a pathogenic role in (severe) respiratory disease.[69] HBoV has been associated with wheezing in asthmatic children.[70] In general HBoV infection is marked by relatively mild symptoms of the upper respiratory tract such as cough, rhinorrhea and fever. In rare cases it has been associated with lower respiratory tract infection and even respiratory insufficiency.[71] Detection of HBoV is by RT-PCR and the virus can be detected in respiratory as well as gastrointestinal specimens.[72] Diagnostic seroresponses can be used to establish the specific immune response against HBoV

during infection, although the clinical relevance is unclear.[73] HBoV can only be cultured on ciliated primary human epithelial cell-lines, and therefore viral isolation is only used in experimental settings.[74] Treatment of HBoV infections is mainly supportive and no specific anti-viral treatment against HBoV is available. Currently there is not enough epidemiological evidence to drive vaccine development against HBoV.

Parechovirus (2006–2007)

Human parechoviruses (HPeVs) belong to the genus *Parechovirus* of the family *Picornaviridae*. The first HPeVs, serotype 1 and 2, were identified 50 years ago during a summer diarrhoea outbreak in American children.[75] With the introduction of molecular techniques many new serotypes of HPeVs have been identified in the past 15 years in the stool or NPA of children with gastrointestinal and respiratory disease, and in the cerebrospinal RT-PCR is only available for HPeV1-3.[87] Currently amplification and nucleotide sequencing is used to identify specific genotypes in a research fluid of children with meningitis and sepsis-like illness (see **Table 2.4**).[76-79] Every HPeV serotype has its specific epidemiological and clinical features.

All HPeVs infections are very common in children under the age of 1 year and most data are available on HPeV1 and HPeV3.[80] The median age of infection with HPeV1 is 6.6 months, whereas HPeV3 infections occur at a younger age of 1.3 months. There is also seasonal variability in occurrence, HPeV1 in late summer and early winter season, and HBeV3 mostly in summer. HBeV serotype 5 and 6 have also been associated with respiratory tract symptoms.[81-84]

Most HPeVs have are common causes of asymptomatic infection in early childhood and are often found in combination with other viruses, so that the relation with respiratory disease is hard to establish.[80] While the association of HPeV3 with encephalitis, meningitis and neonatal sepsis is widely accepted,[85] for most other serotypes the relationship with disease and specific symptoms is less clear (see **Table 2.4**).[86]

A viral neutralisation assay or culture are time-consuming and not suitable for severe disease such as sepsis and meningitis. Detection by setting. The specific antibody response can be used to demonstrate involvement of HPeV in disease if the virus itself cannot be detected. No antiviral treatment against parechoviruses is currently available and only supportive care is given.

Polyomavirus (2007)

In 2007 two new members of the *Polyomaviridae* family were discovered in samples of patients with respiratory disease. The first of these new polyomaviruses was identified during a large scale molecular virus screening project in respiratory samples from children and named after the Karolisnka institute where it was discovered (KIV).[88] The second was identified in a nasopharyngeal aspirate of a 3-year-old child with pneumonia and named Washington University virus (WUV).[89] Seroprevalence studies show KI in 66 %

Table 2.4 HPeV, discovery and clinical associations

HPeV type	Known since	Clinical associations
HPeV1	1956	Mild gastrointestinal and respiratory symptoms, bronchiolitis, pneumonitis, otitis media.
HPeV2	1956	Mild gastrointestinal and respiratory symptoms, (rare) neonatal sepsis, meningitis, encephalitis
HpeV3	2004	Neonatal sepsis, meningitis,encephalitis (transient paralysis)
HPeV4	2006	Fever, mild gastrointestinal and respiratory symptoms
HPeV5	2006	Fever, mild gastrointestinal and respiratory symptoms (sepsis, Reye's syndrome)
HPeV6	2007	Fever, mild gastrointestinal and respiratory symptoms (paralysis, Reye's syndrome)
HPeV8	2009	Enteritis
HPeV10	2010	Gastro-enteritis

and WU in 79 % of paediatric sera.[46,90] The virus has been detected in 1–5 % respiratory samples worldwide in respiratory samples of young symptomatic children.[91-93] However, in 70–80 % of the cases there was a co- infection with other respiratory viruses, and KIV and WUV have been described in asymptomatic HSCT patients.[94] Based on these results it is difficult to assign symptoms and pathogenicity to both of them and more epidemiological evidence is needed. In most studies the viruses have been associated with both upper and lower respiratory tract infections in children. Detection of WUV and KIV in respiratory samples can be undertaken by RT-PCR. Thus far there is no indication for treatment of either of these viruses nor vaccine development.

Influenza A H1N1 Virus (2009)

In late march 2009 a novel influenza A (H1N1) virus was identified in America. This virus was subsequently recognised as the cause of an outbreak of respiratory illness in Mexico.[95] The novel flu virus showed reassortment of swine, avian and human strains, and appeared to be very infectious between humans.[96]

After the initial detection several other countries reported H1N1 infections. In June 2009 the WHO declared a pandemic with spread over at least two continents. At the start of the pandemic the virus appeared to be very virulent with a high mortality rate, especially in young adults and children.[97,98] However, in the Northern Hemisphere the virus behaved more like a seasonal influenza virus. H1N1 disease had the highest attack rate in young children causing relatively mild disease.[99,100] The pH1N1 was able to outcompete the seasonal flu so that, in the influenza season 2009–2010, over 99 % of the influenza positive isolates in Europe and America were pandemic H1N1 influenza A.[101]

In general the symptoms resembled those of other winter viruses: fever, cough, sore throat, myalgia and headache. Symptoms at presentation for hospitalised patients are

Table 2.5 Symptoms of H1N1

Presentation	Literature[a] (%)
Fever (> 38 °C)	81–94
Cough	69–82
Gastro-intestinal symptoms	8–32
Rhinorrhea	31–62
Diarrhea	8–23
Wheezing	12–25

[a] Libster *et al*.[97], Hackett *et al*. [128], Jain *et al*.[129]

shown in **Table 2.5**. Spread occurs up to 8 days after the start of symptoms, although this may be prolonged in the immunocompromised and children.[102]

H1N1 infection can be diagnosed by RT-PCR on respiratory samples and this appears to be the most sensitive method. In case of high suspicion of H1N1 infection with a negative PCR result, the virus can be cultured or infection proven by documenting seroconversion.[103-105]

During the pandemic of H1N1 were treated with oseltamivir (Tamiflu®) and zanamivir. This treatment reduced the duration of symptoms, the occurrence of otitis media and progression into severe disease, especially when administered early in the course of disease.[106] Also the prophylactic use of anti viral agents is effective in reducing the occurrence of H1N1 infections in exposed individuals. However, oseltamivir and multi drug resistant viruses are emerging.[107] In several countries children have been vaccinated.[108,109] H1N1 vaccination induced an effective and long lasting humoral immune response.[108-110] The vaccine seemed to reduce the risk of infection and decreased severity of disease in children, however because of the rapid spread of the H1N1 pandemic most people were vaccinated during the pandemic making efficacy studies complex.[111,112]

Discussion on Molecular Diagnostics of Respiratory Viruses and Their Clinical Use

In this review we have discussed newly identified and emerging viruses from the past 2 decades. These viruses could be subdivided in three categories, based on the evidence for their pathogenicity in respiratory disease in children. First, emerging viruses causing epidemics with high mortality, such as AIV, Hendra and Nipah virus, were clearly associated with a pathogenic role in disease. These epidemic-causing viruses are often of zoonotic origin (transmission from animals to humans). The second group comprises viruses that fulfil the modified Koch's postulates.[8,9] Most novel respiratory viruses are not completely characterised according to the postulates due to the extensive and costly research needed to achieve this. In this perspective hMPV is unique among the recently discovered respiratory viruses, because all criteria have

been fulfilled.[38,113] Third are viruses that were found during screening for new respiratory viruses in respiratory samples with molecular techniques, such as human bocavirus, the novel polyomaviruses, parechoviruses and some coronaviruses.[114] For most of these viruses their pathogenic role as an important respiratory pathogen is less clear. Although these viruses are present in respiratory samples of children with respiratory disease however, they are also often present in asymptomatic children or found in combination with other viruses. Many studies were performed retrospectively, or without the proper control cohorts of asymptomatic children. In epidemiological studies based on seroconversion it is apparent that a first encounter with these viruses occurs early in childhood without (severe) respiratory tract infections. Especially in this last category of viruses, in which the association with respiratory disease is less clear, large prospective epidemiological studies are needed to further specify the pathogenicity and health burden of these viruses in children.

The highly sensitive molecular techniques for identification and detection of novel viruses are a powerful tool for epidemiological studies, especially when used in multiplex platforms. Their ability to quantify the viral burden in infection may be used as additional information in determining the role of a virus in respiratory disease. For some viruses a positive correlation between viral load and disease severity is described.[69 115] However whether viral load correlates with disease severity in general remains a point of debate. Viral load appears to be lower in viral- viral co-infection compared to viral-mono-infections, the mechanism behind this and the clinical relevance requires further investigation.[116,117] Studies show that viral load decreases during the course of disease, and this can be used as marker for the therapeutic effect of anti-viral compounds. A drawback of the high sensitivity of molecular diagnostics is that PCR signals remain positive after recovery from an illness, sometimes even for several weeks. Because young children have frequent viral infections of the upper respiratory tract, the value of a positive PCR test can be limited.

Interaction of viruses with bacteria present in the nasopharynx can result in enhanced disease severity. This is well known for influenza and *Streptococcus pneumoniae*, and other respiratory bacteria.[118] How other (novel) respiratory viruses interact with bacteria and how this leads to enhanced disease is less well known. In studying the pathogenicity of viruses these interactions should be taken into account and implemented in new epidemiological studies. In this context 'old' viruses, like rhinovirus, can be seen in a new perspective and the causality with severe respiratory disease should be re-evaluated.[118-120]

The introduction of molecular detection of viruses has led to the discovery of many new human respiratory viruses and improvement in diagnostics. Novel molecular techniques, like sequencing of the complete virome, will offer new insight in viral infections but also new challenges in proving causality in human disease.

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3

H1N1 infections in Dutch children

Impressions from a university clinic

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Abstract

A discrepancy has been noted in the severity of the clinical course of H1N1 infections between countries of the Northern and Southern Hemisphere. In this article, we retrospectively review our experience with respect to the clinical epidemiology of H1N1 infections in children admitted to a our university hospital. The largest part of the 24 admitted children with proven HiN1 infection was aged > 5 years (71%) and had a severe course of disease (47%). A remarkable high percentage of underlying diseases was noted in these hospitalized children (79%), with most frequent congenital heart disease, followed by chronic lung disease, malignancies and/or immunodeficiency. However, most acute life-threatening presentations of disease occurred in previously healthy children under the age of 3 years.

Introduction

The new Influenza A (H1N1) virus caused an epidemic in the Netherlands from 23 October till 24th of 2009.[1,2] A pandemic of influenza A viruses can occur after an antigenic shift. Influenza viruses are recognised by the immune system by heir hemagglutinin (H) and neuraminidase (N) – antigen. Replacement of the influenza A antigen can occur when several influenza strains infect one single host. Such acute and large changes in the genomic backbone of the virus is called antigenic shift and mostly occurs in the H antigen. In June 2009 the World Health Organisation (WHO) declared the New Influenza A (H1N1) pandemic.[3] After the initial identification of the novel Influenza A H1N1 virus in infected Mexican patients, the virus spread rapidly. Characteristic aspects of this pandemic were the fast spread of the virus and quick notifications on the course of infections in the Southern Hemisphere trough scientific reports and other media. The information from the Southern Hemisphere reached the Netherlands in the summer and raised many concerns. The National Institute for public health and the environment (RIVM) and the Ministry of health, welfare and Sport (VWS) introduced policy measures after consultation with national and international experts. In addition to public information and education to professional organizations, increased hygiene and isolation measures were introduced. In addition, large numbers of vaccines were purchased and guidelines were published on the use of Oseltamivir. Oseltamivir (Tamiflu) inhibits the action of neuraminidases and thereby influences the duration of illness.[4-6] A recent meta-analysis on the use of neuraminidase inhibitors in children demonstrated that the use of Oseltamivir in children is safe.[6]

The H1N1 vaccination campaign started on November 9th in Netherlands for predefined risk groups. The Health Council of the Netherlands advised to extend the vaccination campaign to children between 6 months and 5 years of age and to housemates of children less than half a year on 23 November 2009.[1,2,7] It is now becoming clear that the H1N1 pandemic in the Southern Hemisphere resulted in a more severe course of disease than in the Northern Hemisphere.[8-16] It is important to assess the morbidity and mortality as a result of the pandemic, especially since the clinical aspects of H1N1 infections in children are still relatively unknown. This article describes the clinical epidemiology of H1N1 infections in children in a Dutch university medical center during the H1N1 outbreak in the autumn of 2009.

Patients and methods

This is a retrospective study, although part of the information was prospectively collected. The demographic and clinical data (including medical history, vaccination status, course of disease and outcome) of 61 children from 0 to 18 years who were admitted with the suspicion of a H1N1 infection were gathered by means of a case report form (CRF). From all children a nasopharyngeal swab was obtained and tested

for the presence of H1N1 Influenza A virus by means of a 'quantitative real-time reverse transcriptase polymerase chain reaction (RT-qPCR).[17] Based on these results patients were divided into an H1N1-positive or-negative group. A further subdivision of these groups was based on severity of disease (mild, moderate and severe). Mild disease meant admission without need for supplemental oxygen therapy or tube feeding, patients with moderate disease needed supplemental oxygen or tube feeding and those with severe disease needed mechanical ventilation or died due to the H1N1 infection.

Results

A total of 154 children with symptoms of flu (influenza-like-illness) were tested for an Influenza A (H1N1) infection. Sixty-one children were admitted under the suspicion of a H1N1 infection. Of this group, 24 children (39%) tested positive for H1N1. Two children were not primarily admitted for the suspicion of an H1N1 infection. They were tested because of fever and respiratory complaints during pre-operative screening (n = 1) and during chemotherapy (n = 1). Demographic data and underlying conditions of the study populations are shown in **Table 3.1**.

Table 3.1 Demographic characteristics and underlying conditions in admitted children with (Note: more than one underlying disorders possible).

	Admitted children n = 61 (%)	**H1N1 + n = 24 (%)**	**H1N1 - n = 37 (%)**
Average age in years (range)	5.3 (0.03 to 17.8)	7.9 (0.1 to 6.7)	3.8 (0.03 -17.79)
number of boys	37 (61%)	15 (63%)	22 (59%)
Underlying disease	40 (66%)	21 (79%)	19 (57%)
- asthma	8 (13%)	3 (13%)	5 (14%)
- congenital heart defect	6 (10%)	6 (25%)	0
- Immune suppression / malignancy	7 (12%)	2 (8%)	5 (14%)
- syndrome	3 (5%)	2 (8%)	1 (3%)
- Metabolic / Diabetes	3 (5%)	1 (4%)	2 (5%)
- Preterm birth	3 (5%)	2 (8%)	1 (3%)
- Other	10 (16%)	5 (21%)	5 (14%)

Two-thirds of the admitted children had an underlying medical condition. Congenital heart defects, asthma, malignancies and/or immune deficiency were the most common conditions. Reasons for admission of the H1N1-positive group were age (n = 4), underlying diseases (n = 5), and severity of clinical presentation (n = 15). A subdivision in age groups and severity of disease in H1N1 positive and negative children is shown in **Table 3.2**.

Table 3.2 Age distribution in relation to the severity of disease in H1N1-positive and -negative patients.

	Total		0-1 years		1-2 years		2-5 years		5-19 year	
Disease severity	H1N1 +	H1N1 -	H1N1 +	H1N1 -	H1N1 +	H1N1 -	H1N1 +	H1N1-	H1N1 +	H1N1-
Mild	13	30	3	9	1	3	0	11	9	7
Moderate	3	5	0	1	0	0	0	3	3	1
Severe	8	2	1	0	0	0	2	1	5	1

In November there was a peak in admissions, that same month the first H1N1 vaccinations were administered to patients in risk groups. Vaccinations for children aged between 6 months and 5 years started 2 weeks later. Between August and October 2009 5 children with a H1N1 infection were admitted and in December there were 3 children. One patient was vaccinated a week for presentation in the hospital and the parents of a neonate were vaccinated before the week before the child got ill.

Symptoms

H1N1-positive children often experienced fever (83%), cough (71%) or a combination of these 2 symptoms (63%), see **Table 3.3**. Over 40% of the children experienced gastro-intestinal complaints, diarrhoea (38%) was reported more often than vomiting (21%). They were always accompanied by coughing and/or fever. Two children (8%) presented after a resuscitation situation at home, 1 child (4%) had a circulatory insufficiency at the emergency room. Four children (17%) developed a respiratory or circulatory insufficiency later in the course of disease and were transferred to our hospital. In total, 29% (n = 7) of the children needed mechanical ventilation.

Co-infections

Fifteen out of the 24 children (63%) were tested for co-infections. In 9 of these 15 children (60%) a co-infection was found, 5 viral co-infections were identified (respiratory syncytial virus (RSV), adenovirus, rhinovirus, bocavirus, norovirus) and in five children a bacterial co-infection was detected (*Streptococcus pneumoniae* (sputum), *Streptococcus pyogenes* (empyema), *Acinetobacter baumannii* (blood), *enterococcus faecium* (blood, urine) and *Staphylococcus ugiant* (blood). In 7 of these children with a proven co-infection the course of disease was severe of which 2 patients died.

Course of disease

The average duration of hospitalization for H1N1 positive children was 6 days (1-23 days). Two children with a severe and complex course of disease due to an underlying illnesses were admitted for more than a month and were not included in the average length of stay. All children were treated with Oseltamivir. Antibiotics were prescribed in

Table 3.3 Clinical symptoms at presentation of children admitted with an suspected H1N1 infection

	Admitted n = 61 (%)	H1N1 + n= 24 (%)	H1N1 - n = 37 (%)
Fever	46 (75%)	20 (83%)	26 (70%)
Fever and respiratory symptoms	32 (52%)	15 (63%)	17 (46%)
Cough	31 (51%)	17 (71%)	14 (38%)
Gastrointestinal symptoms	10 (16%)	10 (42%)	4 (11%)
Vomiting	7 (12%)	5 (21%)	2 (5%)
Diarrhoea	12 (20%)	9 (38%)	3 (8%)
Respiratory / circulatory failure	8 (13%)	7 (29%)	1 (3%)
Fever without respiratory symptoms	6 (10%)	2 (8%)	4 (11%)
laryngitis subglottica	5 (8%)	1 (4%)	4 (11%)
Upper Respiratory symptoms	1 (2%)	0 (0%)	1 (3%)
Other	13 (21%)	4 (17%)	9 (24%)

54% of the patients. When looked at severity of disease, 15% of the children with a mild course of disease and all patients in the moderately and severely ill group received antibiotics. The percentage of seriously ill children was relatively higher among older children (5 out of 17 group> 5 years). Patients in the youngest age group were often admitted for observational purposes. All patients with H1N1 infections in the age group of 2-5 years experienced a severe course of disease.

Outcome

Four patients (17%) deceased. Two patients, both younger than 4 years, died in the ICU after resuscitation at home. In one of them both a bacterial and a viral co-infection was detected. Another deceased patient refused intensive care treatment due to his underlying disease and with the consent of his parents and physicians this was accepted. The fourth patient, where there was also a viral co-infection, died due to his underlying disease during an H1N1 infection. The last 2 patients were both older than 5 years.

Discussion

The H1N1 epidemic caused an increase in consultations and admissions that peaked in November 2009. In particular the confrontation with resuscitations of young, previously healthy children in the home setting caused concerns. This led to a low threshold for admission of children <5 years of age with the suspicion of H1N1 infection. In retrospect the severity of disease due to H1N1 was relatively mild, although there are some notable observational done.

Of all admitted children with a H1N1 infection the majority were older than 5 years of age (17 of the 24 children, 71%) and it was especially in this age group that there was a serious course of disease (8 of the 17 children, 47%). A Canadian study in which 58 children with H1N1 infection are described, shows a similar distribution in terms of age distribution and severity of disease. Sixty-four percent of the children with H1N1 infection was older than 5 year, a significant difference with respect to the children who were before them in 5 years recorded with a seasonal Influenza A infection.[18] Initial reports from the United Kingdom show a similar distribution, where 12 of the 13 children who were included in intensive care with H1N1 was older than 5 years of age. [9,11]

The Dutch data collected by the National Institute for public health and the environment (RIVM) showed that the number of admissions per 100,000 is highest for the age group 0 to 5 years (62.7).[1,2,7] The proportion of children under 6 months of age in this whole group is 33%. This percentage might be positively influenced by the advise to admit children <3 months during treatment with Oseltamivir. The number of admissions to intensive care units per 100,000 for the age group 0-5 years is equal to the number of ICU admissions for 5 to 14 years old (1.73 and 1.71, respectively) while the mortality in the youngest age category is higher per 100,000 (0.54 vs. 0.45, respectively). When we look at absolute numbers, more young children from 0 -4 years (581 vs. 384) were admitted to the hospital, but twice as many children aged 5 to 14 years needed admission to the ICU (32 vs. 16). Finally more children deceased in the age group 5 to 14 years (9 vs. 5).[1,2,7]

A number of exceptionally serious manifestations of H1N1 infections in previously healthy children were observed. Two children needed to be resuscitated at home, another arrived in circulatory shock at the emergency room. These three children were all 3 years old or younger, and represented 43% of all admitted children in the age group < 5 years of age; the other children from this age group (n = 4) were only admitted for observational purposes. In the study of Libster et al. a death rate of 5% is found this is significantly lower than the 17% mortality rate in our population. The number of ICU admissions (33 versus 19%) and the number of children that needed mechanical ventilation (23 versus 17%) is also higher in our population.[10] Miroballi *et al.* showed a similar rate of ICU admissions in New York, but they described a lower percentage of mechanical ventilated children and a lower mortality rate of 1%. [14] This might be due to the academic setting of the hospital resulting in a bias that results in more children with significant comorbidity. Further the hospital functions as tertiary referral centre for the region.

The incidence of fever (83%) and cough (71%) as presenting symptoms of H1N1 infections in children is similar to the symptoms found in the literature (respectively 84-92% and 69-91%).[9,10,19]

However, there are also striking differences between our study and previously published studies concerning the presence of rhinitis and upper respiratory tract symptoms (ranging from 31-77% vs. 0% in our study group) and the occurrence of gastro-intestinal complaints (ranging from <10% to 37% versus 42% in our study). [9-11,19]

The influence of co-infections in the course of disease and the outcome is not yet clear. Libster *et al*. mention a (suspected) bacterial pneumonia in 10% of the children with a H1N1 infection, of which 16% developed an empyema. In 7% of the tested children (n = 147) a positive blood culture was found and 19% had a viral co-infection, usually RSV (89%). Four children with a proven co-infection deceased in this group.[10] Miroballi *et al.* found no viral co-infections and 3.5% bacterial co-infections. [14] These two studies show no increase in mortality based on the presence of a co-infection.[10,14]
In our study, children with H1N1 infections were not routinely tested for co-infections. Ultimately, 63% of the hospitalized children were tested for co-infections. The choice to perform diagnostics in children was based on the clinical parameters of the individual patient. Seventy-five percent of the patients with a severe course of disease had a viral or bacterial co-infection, also in 2 of the 4 deceased children a co-infection was demonstrated. Based on these small number of patients it is not possible to make a statement about the impact of co-infections in the course of disease.
Underlying medical conditions are a main risk factor for hospitalization and death due to influenza infections. Over 70% of the admitted children with a H1N1 infection had an underlying disease (chronic lung disease, immune deficiency, cardiac and neurological diseases). Other studies published lower percentages (40.3% and 34%) of underlying diseases in comparable paediatric populations.[9,11] The function of our hospital as a tertiary referral centre as previously mentioned might explain the differences in the percentage of children with an underlying medical condition. In our study there was no difference in mortality observed in patients with and without an underlying disorder. Fifty percent (2 out of 4 deceased children) had an underlying condition. In an Argentine study the presence of a complex underlying disease was significantly more frequent in a group of children who died due to an H1N1 infection in the intensive care.[20]

Conclusion

The description of the clinical epidemiology of H1N1 infections in Dutch children admitted to our university hospital revealed a number of striking observations: 1) most children admitted with a H1N1 infection were older than 5 years of age (71%); 2) in this age group the course of disease was more often severe (47%); 3) a remarkably high percentage of the children with H1N1 infection had an underlying disease (79%); in particular congenital cardiac disorders were found, but also asthma, malignancies and / or immune deficiency were frequent; 4) the most acute (life threatening) presentations occurred in previously healthy children under 3 years of age.

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4

Nationwide study on the course of influenza A (H1N1) infections in hospitalized children

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Submitted

Abstract

Background: the influenza H1N1 pandemic of 2009-2010, provides a unique opportunity to assess the course of disease, as well as risk factors for severe disease in Dutch children (<18 years) hospitalized with influenza- virus infections.
Methods: retrospective national chart study on children admitted to a Dutch hospital with a RT-PCR confirmed H1N1 infection during the 2009-2010 pH1N1 outbreak.
Results: 940 patients (56% boys), median age of 3.0 year [IQR 0 - 9 years] were enrolled. The median duration of hospitalization was 2.0 days [IQR 1-4 days]. Of these children, 63% received antiviral treatment, 24% needed supplemental oxygen, 7% were admitted to the ICU and 5% needed mechanical ventilation. *Staphylococcal aureus* was the most common bacterial co-infection. Fifteen patients died (1.6%), five were previously healthy. After multivariable analyses risk factors associated with intensive care admission were pre-existent heart and lung disease. Risk factors for mortality were neurologic conditions and an oncologic medical history. At least 1 vaccination was administered to 66 patients at the peak of the pandemic. One third of these children (n=23, 35%) were hospitalized within 1 week. We did not observe a clear effect of vaccination due to the limited number of patients with a known vaccination status and due to the introduction of vaccines at the peak of the pandemic.
Conclusions: This study presents a nationwide overview of Dutch hospitalized children during the 2009 H1N1 pandemic and reconfirms known risk groups for severe influenza infections. Most of the acute and severe presentations occurred in previously healthy children.

Introduction

The 2009-2010 influenza A (H1N1) outbreak offered a unique possibility to assess the impact of infection with this pandemic virus in terms of clinical features and disease outcome in children. The early detection of H1N1, the rapid worldwide dissemination of information and the expectation of a severe course of disease raised concerns about a global outbreak with substantial morbidity and mortality in the Netherlands.[1,2] The absence of herd immunity due to antigenic shift of the virus increased the risk for severe disease in the population and particular in risk groups for influenza who would not be protected by the seasonal influenza vaccine. In retrospect, only the elderly appeared to have some pre-existing immunity.[3] The fear of a severe pandemic resulted in a nationwide protocol using RT-PCR in adults and children with (severe) manifestations of influenza-like illness, as defined by the WHO.[4] To date the course of disease in pH1N1 infections has been described by many investigators. However, data on children remain underreported, while children comprise more than half of all infected patients worldwide during this pandemic.[5]. We therefore evaluated disease characteristics in the 2009-2010 influenza A (H1N1) pandemic among all hospitalized children in the Netherlands in order to increase our understanding of the clinical features of hospitalized children, to assess the presence of risk groups for severe disease and to study protection provided by H1N1 vaccination.

Methods

A retrospective nationwide analysis of clinical case records was performed of RT-PCR confirmed H1N1-infected children (<18 years) admitted for at least one night to Dutch hospitals between August 1, 2009 and August 1, 2010. Diagnostic RT-PCR was carried out in the participating hospitals in The Netherlands, with a protocol standardized and approved by the National Influenza Center.[4] Children who tested positive for Influenza A, but not for pandemic influenza H1N1 were excluded. Children who tested positive, but were admitted for another reason, e.g. elective surgery, were also excluded. Approval of the local medical-ethical committees was obtained from each hospital. Clinical files were studied by one of the investigators (RP, ML, IA or GF). In situations of doubt regarding interpretation of data, a second investigator was consulted and a shared decision about interpretation was recorded. Clinical, laboratory, and radiological data were obtained and abstracted into a clinical record form. For chronically ill patients with prolonged hospitalization due to other causes than pandemic influenza H1N1, the symptomatic respiratory period was used to determine duration of hospitalization.
Data were entered into a database by one investigator (MB) and subsequently checked by another (IA, RP, or ML) to exclude errors. Data are presented as numbers (percentages) or median with inter-quartile range (IQR). Clinical symptoms, treatment, and medical history were compared among different age groups (<6 months, 6-24 months, >2 years)

with Kendall's Tau statistics for trend analysis. Kruskall Wallis, Chi-square and Mann Whitney U-test were used to test for differences between groups. Gestational age was missing in 221 (24%) of the cases. Therefore, analyses were repeated in patients in whom the preterm status was known. For all other variables the percentage of missing data was <10%. Binary logistic regression analysis was used to identify risk factors that were associated with a binary outcome measure, e.g. mortality, ICU admission, or prolonged hospitalization of > 7 days. We calculated both unadjusted and adjusted odds ratios (ORs) with 95% confidence intervals (CIs) with the latter including all other potential risk factors. All data were analyzed using SPSS version 22 (SPSS Inc., Chicago, IL, USA).

Results

Seventy-five of the 91 hospitals throughout the Netherlands participated in this study (82%), including all tertiary centers and all large teaching hospitals. Four hospitals intended to participate, but did not have eligible patients. Seventeen hospitals refused to participate. Reasons for non-participation were: ''too much effort for only a few patients'' (n=14), logistical problems (n=2) or because they did not test for H1N1 specifically (n=1). An average of 12 patients [range 0-69] were included per hospital (**Figure 4.1**). From 62 hospitals, information regarding the total number of positive H1N1 RT-PCRs was available, resulting in a hospitalization rate of 35% (731 hospitalizations among 2065 H1N1 positive samples).

In this study 940 patients were included. The hospitalization rate was 28.4 per 100.000 (940 / 3,312,432). The highest rate of admissions was seen in week 46 with 188 hospitalized children (**Figure 4.2a**). The number of children admitted to the ICU showed a peak in week 45. The age distribution of these patients is shown in **Figure 4.2b**. The largest number of admissions was documented in infants below 1 year of age (140.9 per 100,000), followed by infants 1-2 years of age (60.5 per 100,000). The number of admissions in older children remained almost equal for children up to 7 years of age (between 20-30 per 100,000) and decreased in those older than 7 years (<15 per 100,000). The incidence of hospitalization (including ICU admission) was 57.4 per 100,000 in 0-4 year old children, 17.6 per 100,000 in 5-14 year old children and 9.80 per 100,000 children in children aged 15-18 year.

The majority of children with influenza H1N1 infection had fever (89%, defined as temperature >38˚C), followed by cough (71%). The proportion of cough appeared to increase per age group from 61% to 75% ($p<0.01$). Almost one third of patients experienced dyspnea. The presence of dyspnea increased with age. Gastrointestinal symptoms were more frequently seen in patients between 6 months to 2 years of age as compared to infants below 6 months and above two years. Neurological complications were diagnosed in 10% of patients, especially in those between 6 months and two years of age (15%). This was significantly higher compared to both other age groups (**Table 4.1A**).

The most common co-morbid condition was wheezing or asthma, followed by neuromuscular conditions and prematurity at birth. The frequency of co-morbidity increased with age (**Table 4.1B**). Positive bacterial cultures were found in 6% of all admitted children (59 positive out of 940 patients), although only 36% of the patients were tested for a bacterial co-infection (338/940). In the patients that were tested 17% had a bacterial co-infection (59/338). Staphylococcal co-infection was most frequently detected (in 13 out of 59 positive patients, 22%), followed by *E. Coli (n=4*The majority of patients received antiviral therapy (63%). Eight patients received zanamivir. Reasons to give zanamivir or to switch from oseltamivir to zanamivir were liver failure, last resort since

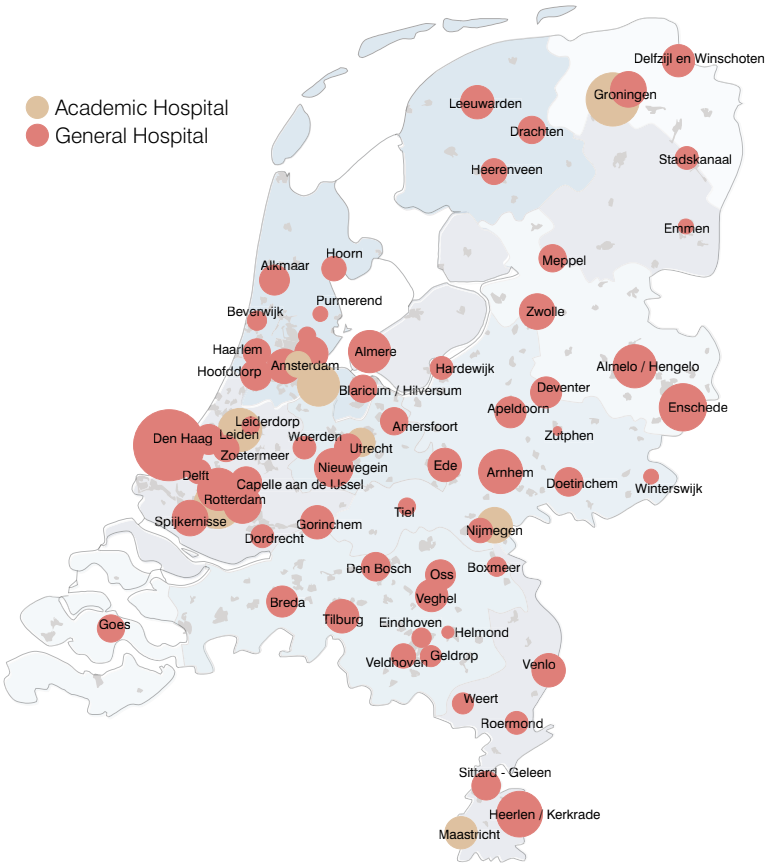


Figure 4.1 Regional representation of participating hospitals and number of children included per site. The red / dark circles represent general hospitals and the light circles are the academic medical centres (n=8). The size of the circles represents the number of included patients per site.

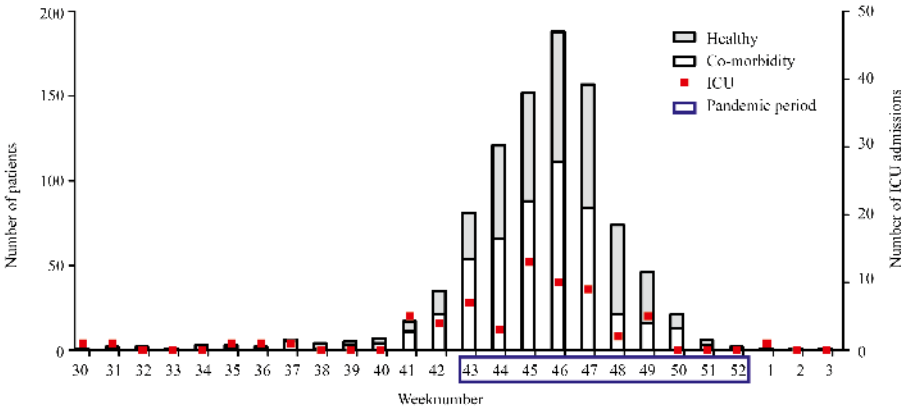


Fig 4.2a Number of hospitalizations per week. The height of the bar represents the number of admissions per week. Colours differentiate between healthy children and children with co-morbidity. Red dots represent the number of children admitted to the intensive care unit (right axis).

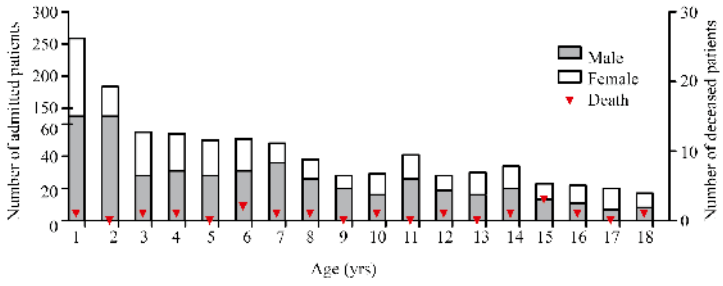


Fig 4.2b Age and gender distribution of admitted children. The height of the bar represents the number of admissions per age group. Colours differentiate between genders. Red triangles represent the number of children who died due to pH1N1 infections (right axis).

patient deteriorates (n=5). Frequently noted reasons to abstain from antiviral treatment were: duration of symptoms >48 hours or mild course of disease. Oseltamivir was the only treatment of which the frequency decreased amongst the age groups (**Table 4.1B**). All other treatment modalities, including oxygen and antibiotics were significantly more applied in older patients. Eighty-seven patients (9%) were hospitalized longer than a week. Longer hospitalizations were more often seen in the older age groups. Sixty three patients (7%) were admitted to the ICU, 18 (3%) were previously healthy and 5% needed mechanical ventilation. Six patients were treated with ECMO. In this study a total of

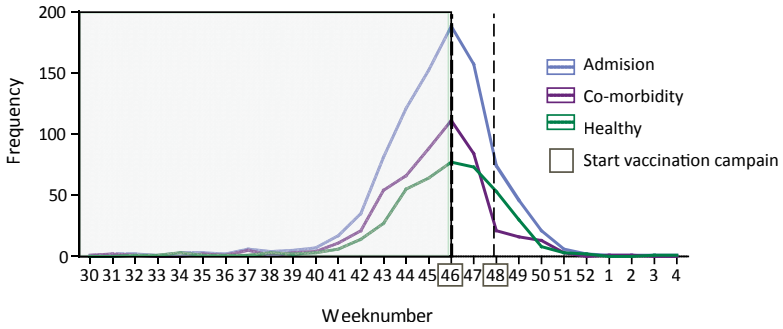


Figure 4.2c Number of hospitalizations per week in relation to start vaccination campaign. Frequency curves represent the total number of admissions per week (blue), the proportion of children with a co-morbid condition (purple) and number of previously healthy children (green). Vaccination started in week 46 for risk groups, in week 48 the target group was extended to all children between 6 months and 4 years of age and household contacts of children below 6 months of age.

fifteen patients died (1.6%). Eleven of them died in the ICU department. Five were previously healthy, including the two patients who received ECMO both were previously healthy, > 12 years old and suffered from a co- infection with *Staphylococcus aureus*. Two previously healthy patients (<5 years) died at home after resuscitation. One had an adenovirus co-infection, the other had a positive sputum sample for *Staphylococcus aureus* with bilateral consolidations on the chest X-ray. This patient died as a consequence of brain edema and encephalitis. The fifth previously healthy patient (<15 years) died of a bacterial co-infection. The chest X-ray showed bilateral consolidations. None of the patients who died were below 6 months of age. Patients younger than 6 months were less likely to need supplemental oxygen or tube feeding while they were more often treated with antiviral medication compared to both other age groups (**Table 4.1B**).
In week 46 the H1N1 vaccine became available for risk groups. At that moment the H1N1 pandemic was at its peak (**Figures 4.2a** and **4.2c**)**.** Two weeks later the vaccine indication was extended to household contacts of children < 6 months of age and all children up to 4 years of age. Sixty-six patients received at least 1 pH1N1 vaccination, one third (n=23, 35%) received the vaccine within one week of hospitalization. This resulted in 43 (partially) vaccinated infants. None of these children died. None of the patients with a bacterial co-infection was previously vaccinated with the pH1N1 vaccine, although 3 patients received a vaccine within a week before hospitalization; their positive blood sample appeared to be contaminated. Six were admitted to the intensive care and three needed mechanical ventilation. Since this is a retrospective chart study we were unable to retrieve information on H1N1 vaccination status in many patients. **Flowchart 4.1** shows the distribution of the hospitalized patients in time and the information regarding immune status. In **Figure 4.2c** the admission rates of children

Table 4.1 Patient characteristics

A Clinical symptoms

	All (n=940)		< 6 months (n=171)		6 months - 2 years (n=201)		>2 years (n=568)		*p*-value	Kendall's tau
Symptoms	**n**	**%**	**n**	**%**	**n**	**%**	**n**	**%**		
Fever (>38 C)	840	89	153	90	184	92	503	89	NS	*-0.026*
Cough	667	71	105	61	137	68	425	75	$p<0.01$	*0.133*
Rhinorrhea	324	35	81	47	103	51	140	25	$p<0.001$	*-0.241*
Dyspnea	289	31	23	14	54	27	212	37	$p<0.001$	*0.193*
Vomit	280	30	36	21	60	30	184	32	$p<0.05$	*0.083*
Diarrhoea	144	15	24	14	51	25	69	12	$p<0.05$	*-0.081*
Dehydration	47	5	4	2	14	7	29	5	NS	*0.022*
Convulsion/neurologic disorder	91	10	1	1	30	15	60	11	$p<0.001$	*-0.197*
Decreased consciousness	32	3	4	2	4	2	24	4	NS	*0.053*

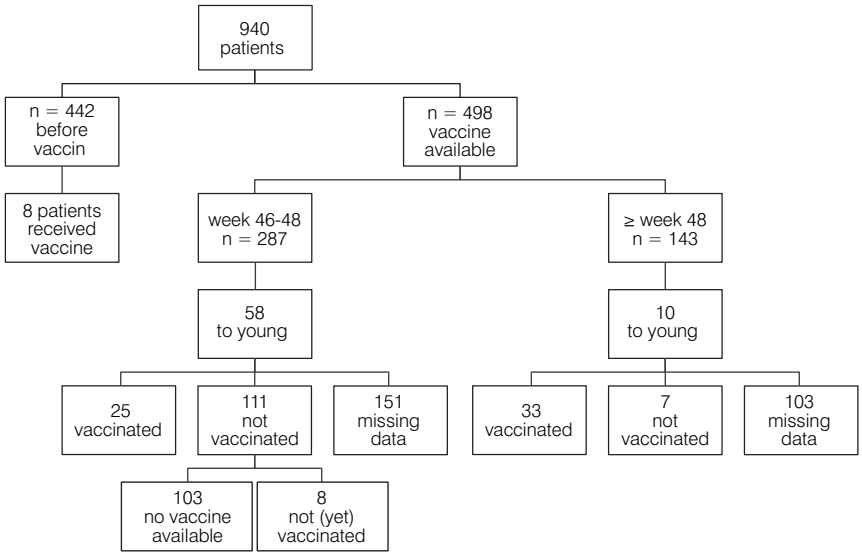
B Co-morbidities, treatment and outcome

	All ages (n=940)		< 6 months (n=171)		6 months - 2 years (n=201)		>2 years (n=568)		*p-value*	Kendall's Tau
Medical history	**n**	**%**	**n**	**%**	**n**	**%**	**n**	**%**		
Boys	528	56	81	47	124	62	323	57	NS	-0.37
Healthy	427	45	142	83	110	55	175	31	**<0.001**	**-0.378**
Asthmatic / atopic condition	255	27	13	8	35	17	207	36	**<0.001**	**-0.256**
Neurologic condition	128	14	1	1	16	8	111	20	**<0.001**	**-0.214**
Prematurity	103	11	20	12	34	17	49	9	NS	-0.007
AD missing	221	24	0	0	7	4	214	38		
SV/malnutrition	52	6	2	1	9	4	41	7	**<0.01**	**-0.095**
Immunodeficiency	52	6	2	1	5	2	45	8	**<0.001**	**-0.124**

Lung condition	50	5	5	3	5	2	40	7	**<0.01**	**-0.087**
Cardiac condition	38	4	2	1	9	4	27	5	NS	-0.054
Metabolic / endocrine disease	32	3	0	0	5	2	27	5	**<0.05**	**-0.095**
DM	16	2	0	0	1	0	15	3	**<0.01**	**-0.086**
Oncologic disease	16	2	0	0	0	0	16	3	**<0.01**	**-0.099**
Down syndrome	10	1	1	1	2	1	7	1	NS	-0.022
Sickle cell disease	9	1	0	0	0	0	9	2	**<0.05**	**-0.074**
Treatment										
Antiviral	590	63	125	73	127	63	338	60	**<0.01**	**0.093**
Antibiotic	382	41	39	23	61	30	282	50	**<0.001**	**-0.233**
Oxygen	224	24	21	12	40	20	163	29	**<0.001**	**-0.144**
Tube feeding	212	23	17	10	58	29	137	24	**<0.05**	**-0.080**
Mechanical Ventilation	43	5	2	1	3	1	38	7	**<0.001**	**-0.117**
ICU	63	7	3	2	5	2	55	10	**<0.001**	**-0.139**
>7days	87	9	8	5	16	8	63	11	**<0.05**	**0.081**
Outcome										
Discharged	925	98	171	100	200	100	554	98	**<0.05**	**0.068**
Deceased	15	2	0	0	1	0	14	2	**<0.01**	**-0.082**

Comparisons between three age groups were perfomed with Kendall's Tau statistics

Flowchart 4.1 Description of cohort in relation to vaccine introduction of pH1N1 vaccination.



with and without a vaccine indication in week 46 are shown separately, in this figure the number of hospitalizations in children of risk groups appears to decrease earlier and steeper compared to healthy children.

Co-morbid conditions were analyzed to identify risk groups for a severe course of disease, defined as ICU admission, prolonged hospital stay of more than 1 week or mortality. Cystic fibrosis, Down syndrome and sickle cell disease could not be analyzed in the model since the number of patients with these conditions was too low (<20 cases). Due to missing information regarding prematurity this risk factor was left out of the initial analysis. Results of these analyses are shown in **Table 4.2A**. Risk factors for prolonged hospitalization: any co-morbid condition (OR 1,9 95% 1.1-3.3), neuromuscular disease or psychomotor retardation (OR 2.3, 95%CI 1.3-4.2), malnutrition, including need for tube feeding (OR 2.3, 95%CI 1.1-4.7) and heart disease (OR 2.6, 95%CI 1.2-5.9). Including age to the model did not influence these results. The same analyses were performed for need for ICU admission: pre-existing heart disease (OR 3.3, 95%CI 143-8.0) and lung disease (OR 2.4, 95%CI 1.0-5.5) were identified as risk factors. After correction for age only heart disease (OR 3.0, 95%CI 1.3-7.2) remained a significant risk factor. Mortality was associated with a medical history of neuromuscular disease or psychomotor retardation (OR 4.3, 95%CI 1.5-12.4) and oncologic disease (OR 9.6, 95%CI 1.9-48.4). Age was no risk factor in this outcome measure. When the same analysis was performed in a subgroup of patients in which pre-term status was known

Table 4.2 Comparisons between the three outcome measures, prolonged hospitalization, ICU admission and mortality in uni- and multivariate analysis

	>7 days			ICU			Mortality		
	Unadjusted OR (95% CI)	Adjusted OR (95% CI)	Adjusted for age groups OR (95% CI)	Unadjusted OR (95% CI)	Adjusted OR (95% CI)	Adjusted for age groups OR (95% CI)	Unadjusted OR (95% CI)	Adjusted OR (95% CI)	Adjusted for age groups OR (95% CI)
Gender	0.9 (0.6-1.4)			1.1 (0.7-2.0)			0.5 (0.2-1.5)		
Not healthy	**1.5 (1.3-1.7)**	**1.9 (1.1-3.3)**	**1.9 (1.1-3.3)**	**1.3 (1.1-1.5)**			1.4 (0.99-1.8)		
Preterm	1.3 (0.7-2.3)	NA	NA	0.9 (0.4-1.1)	NA	NA	NA*		
Diabetes	2.1 (0.7-5.8)			1.9 (0.5-7.1)			NA *		
Asthma /atopy	0.96 (0.6-1.5)			1.3 (0.8-2.2)			1.3 (0.5-3.9)		
Neurology / PMR	**3.3 (2.2-5.0)**	**2.3 (1.3-4.2)**	**2.3 (1.3-4.2)**	1.6 (0.9-2.9)			**4.2 (1.5-11.7)**	**4.3 (1.5-12.4)**	**4.3 (1.5-12.4)**
Malnutrition	**3.8 (2.4-6.1)**	**2.3 (1.1-4.7)**	**2.3 (1.1-4.7)**	1.5 (0.6-3,5)			**4.2 (1.2-14.7)**		
Heart	**3.0 (1.6-5.3)**	**2.6 (1.2-5.9)**	**2.6 (1.2-5.9)**	**3.0 (1.5-6.1)**	**3.3 (1.4-8.0)**	**3.0 (1.3-7.2)**	NA		
Lung	**2.1 (1.0 - 4.4)**			**2.2 (1.1 -4.6)**	**2.4 (1.0-5.5)**	2.0 (0.9-4.8)	1.3 (0.2-9.5)		
Immunodeficiency	1.7 (0.9-3.4)			**2.1 (1.0 -4.5)**			**4.3 (1.2 -14.7)**		
Down syndrome	1.1 (0.2-7.0)			3.0 (0.9-10.8)			6.6 (0.96-45.8)		
Oncology	2.1 (0.7-5.8)			**2.9 (1.0 -8.2)**			**8.9 (2,1 - 36.2)**	**9.6 (1.9-48.4)**	**9.6 (1.9-48.4)**
Metabolic	2.1 (1.0-4.4)			1.4 (0.5-1.1)			**4.4 (1.0 - 18.5)**		
Age group						**0.3 (0.2-0.6)**			

* None of the patients with preterm birth, diabetes or heart disease died in this study

(n=718), prematurity was not associated with one of the outcome variables from this study (**Supplemental Table 4.1**).
The large proportion of previously healthy children (427 children, 45%) prompted us to further explore this group. The length of stay in the hospital was similar in healthy infants and patients with a medical history. Patients with a medical history did receive more antiviral ($p<0.05$) or antibiotic ($p<0.01$) treatment and were also more likely to need supplemental oxygen ($p<0.001$), tube feeding ($p<0.01$) or mechanical ventilation ($p<0.05$). Patients with a medical history had an OR of 1.4 (95% CI 1.4-1.6) of being admitted to the ICU (n=45/513, 9%) versus previously healthy infants (n=18/427, 4%). Four previously healthy patients died (0.9%) versus 11 (2.1%) patients with a medical history (NS).

Discussion

In this study the H1N1 pandemic of 2009-2010 in the Netherlands was used to gain insight in the course of a pandemic influenza A infection in children, the effect of the vaccination campaign and to identify risk groups for severe influenza infections in children. This study is unique in its national design with a uniform diagnosis via RT-PCR and detailed insight in the course of disease.
The epidemiological data in this study are similar to those in earlier studies from the Netherlands and Europe. This indicates that the national infectious disease notification system worked adequately.[6] Steen *et al.* fount an infection attack rate of around 7.5% [4-11 95% CI] in the general population, with a higher attack rate of 35% in children aged 5-19 years (25-45 95% CI) in a serological study.[7] Van het Klooster *et al.* also show that hospital admission rates were highest in 0-4 year olds, followed by 5-14 and 15-24 year old children, then adults aged 45-54 and subsequently the age groups 25-34 years.[6]
Many studies have documented the course of the pH1N1 infection.[6,8-13] Compared to other studies the length of hospitalization in the Netherlands was relatively short [8,10,13] or similar.[12] The percentage of ICU admissions was slightly lower (7% versus 8.8-27%) [13-19] and the proportion of deaths slightly higher (2% versus 0.5 -3.0%) than in previous publications .[14-16,20,21] Although American studies clearly show an excess in pediatric mortality during the winter of 2009/2010 compared to winters with seasonal influenza.[22,23]
Approximately 50% of the patient in this study had significant co-morbidity, which is in agreement with other studies that reported percentages between 40-75%. [13-16,21,24,25]
The effect of the vaccination campaign was difficult to establish, since the introduction of the vaccine coincided with the peak of the pandemic. The vaccine uptake in the second round, starting in week 48, was 75% (n=588.750) for children between 6 months and 4 years of age and 63% for invited household contacts.[26] As shown in figure 2c, there appears to be a steeper decline in the number of hospitalizations of patients with a co-morbidity from week 46 onward compared to previously healthy patients. The effect seems most evident in week 48, when healthy patients are more frequently

admitted to the hospital than patients with a co-morbid condition. However, in this week the number of hospitalizations was low. In our opinion the decline starts very early and based on this retrospective study with limited data on vaccination status it is not possible to draw a solid conclusions on the effect of vaccination. In serological or database driven studies positive effects have been described. [27-29]

Risk factors associated with intensive care admission in this study were pre-existent heart and lung disease. Other studies in which risk factors for a severe course of H1N1 infections were identified had fewer inclusions, but found similar results. [9,11,13,21,30] An Australian study added bacterial co-infection (OR 6.89 95% CI 3.2-15.1) [9] whereas Garcia *et al*. recognized obesity, premature birth and hematological disease as risk factors for severe disease.[11] Stein *et al.* additionally found metabolic disorders to be a risk for intensive care admission.[13] In this study risk factors for mortality were a pre-existent neuromuscular or psychomotor developmental disorder and an oncologic medical history. The increased risk of death due to influenza in children with a chronic neurological condition was also found in a study of Pebody *et al*. They report that 92% of the deceased children with an underlying condition had a neurodevelopmental disorder.[31]

The limited number of co-morbid conditions like cystic fibrosis, metabolic disorders, Down syndrome and sickle cell disease in our study made it impossible to include these conditions in our analyses. This is an interesting finding by itself, since especially Down syndrome and diabetes are common diagnoses with a prevalence of 12 per 10,000 births for Down syndrome and an incidence of 18.6 per 100,000/year diabetes cases, resulting in approximately 5400 and 6000 pediatric patients in the Netherlands.[32,33] This may suggest that children with these diagnoses were not more affected than the general population. In our study prematurity was not a risk factor for a severe course of disease. Gill *et al*. did find an association between prematurity and need for hospitalization for children up to two years of age in an emergency room study.[34] In our study prematurity was only a risk factor for severe disease in a subgroup of children (< 6 months of age) (data not shown).

A large proportion of previously healthy children was admitted to the hospital. The majority was below 2 years of age and admitted for observation. However, five out of the 15 deceased patients were previously healthy and three of them entered the hospital after resuscitation at home. During our study we encountered at least two other infants who died outside the hospital. Since these children were not hospitalized they were not included. [6] Studies describe that 35-43% of deaths through influenza occur outside the hospital or in the emergency room. Death outside the hospital was more likely in children without high-risk medical conditions, as we also observed in this study.[23,35] Shrag et al. performed a surveillance study for influenza hospitalizations in America and described that 4 out of 6 paediatric deaths did not have a medical indication for influenza vaccination. Noteworthy, in their study they could not confirm that the presence of a medical history (vaccine indication) was associated with intensive care admission.[17] In this study stringent inclusion criteria were used. Due to the retrospective nature of this

study, missing data form a limitation. Especially data with regard to vaccination status and prematurity was difficult to find. Moreover, obesity as risk factor was impossible to assess, since especially length of children was rarely recorded. Furthermore, a limitation of our study is the lack of a control group. This would have enabled us to analyze the need for hospitalization as an outcome measure or would have helped us to correct for overrepresentations of conditions in our patient population.

We reported the clinical course of hospitalized Dutch patients with a uniformly RT-PCR diagnosed pH1N1 infections. H1N1 infections resulted in respiratory and neurological symptoms. However, some of the children presented with an acute or very severe disease entity with shock, resuscitation out of the hospital or necrotizing encephalitis. Most of these acute cases occurred in previously healthy children. In this study *Staphylococcal aureus* was the most common bacterial co-infection. Risk groups for a severe course of disease could be identified and overlapped with risk groups of seasonal influenza, although the proportion of healthy children was substantial. Due to the late introduction of the influenza vaccine no clear beneficial effect of vaccination could be observed.

Supplemental Table 4.1 Comparisons between the three outcome measures, prolonged hospitalization, ICU admission and mortality in uni- and multivariate analysis in a subset of patients of which preterm status is known.

	>7 days known preterm status			ICU known preterm status			Mortality known preterm status		
	Unadjusted OR (95% CI)	Adjusted OR (95% CI)	Adjusted for age groups OR (95% CI)	Unadjusted OR (95% CI)	Adjusted OR (95% CI)	Adjusted for age groups OR (95% CI)	Unadjusted OR (95% CI)	Adjusted OR (95% CI)	Adjusted for age groups OR (95% CI)
Gender	0.9 (0.5-1.4)			1.1 (0.6-2.2)			0.4 (0.1-1.5)		
Not healthy	**3.0 (1.7-5.2)**	**2.0 (1.0-4.0)**	**2.0 (1.0-4.0)**	**2.5 (1.2-5.1)**			2.5 (0.7-9.2)		
Preterm	1.3 (0.7-2.3)			0.9 (0.4-2.3)			NA		
Diabetes	1.1 (0.2-7.4)			2.0 (0.3-13.0)			NA		
Asthma /atopy	1.1 (0.7-1.9)			1.3 (0.6-2.5)			1.7 (0.5-5.9)		
Neurology / PMR	**3.4 (2.1-5.5)**	**2.8 (1.5-5.4)**	**2.8 (1.5-5.4)**	**2.6 (1.3-5.1)**	**2.4 (1.1-2.4)**		**5.8 (1.8-18.5)**	**6.0 (1.8-20.2)**	**6.0 (1.8-20.2)**
Malnutrition	**3.5 (1.9-6.3)**			2.2 (0.8-6.0)			4.1 (0.9-18.3)		
Heart condition	**3.5 (1.9-6.4)**	**2.9 (1.2-6.8)**	**2.9 (1.2-6.8)**	**4.0 (1.8-9.0)**	**4.1 (1.5-10.8)**	**3.8 (1.5-10.1)**	NA		
Lung condition	2.2 (1.1-4.6)			**2.8 (1.2-6.8)**			NA		
Immunodeficiency	1.8 (0.8-4.2)			1.1 (0.3-4.7)			2.1 (0.3-15.8)		
Down syndrome	NA			NA			NA		
Oncology	2.6 (0.7-9.0)			2.2 (0.3-14.3)			**7.9 (1.1-55.3)**		
Metabolic	0.9 (0.2-3.7)			NA			2.9 (0.4-21.7)		
Age group						**0.4 (0.2-0.7)**			

* None of the patients with preterm birth, diabetes or heart disease died in this study

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5

Influenza vaccination in kids, are you kidding me?

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Summary

Seasonal influenza infections cause a high burden of disease for the whole community every year. Effective vaccines are available and used worldwide in adults and children. Discussion is ongoing as to whether influenza vaccination for children should be implemented in the National Immunization Program (NIP). Is there enough evidence to support routine influenza vaccination in children? In this review we briefly discuss the influenza viruses and the available vaccines. Subsequently, the current data available on influenza vaccination is reviewed and weighed against the Dutch criteria for the introduction of new vaccines into the NIP.

Introduction

Influenza virus is a RNA virus belonging to the *Orthomyxo- viridea* which primarily infects the respiratory tract. Trans mission mainly occurs through aerosols created after coughing. However, the virus can also be transmitted via direct contact with nasal secretions (daycare centers and siblings) or faeces of animals (bird droppings and avian flu).[1] Influenza occurs worldwide in winter epidemics, resulting in three to five million cases of severe illness and 250,000 to 500,000 deaths yearly.[2] Some years, a new influenza A virus subtype emerges in which the hemagglutinin gene is not related to the circulating influenza strains. Influenza strains can acquire genes from influenza viruses that infect birds or pigs. These new strains of influenza viruses can spreads worldwide and cause a pandemic.[3-5]

In late March 2009 such a novel influenza A (H1N1) virus was identified in Southern America which showed reassortment of swine avian and human strains.[6] This virus was subsequently recognized as the cause of an outbreak of respiratory illness in Mexico.[7] In June 2009 the WHO declared a pandemic. At the start of the pandemic the virus appeared to be very virulent with a high mortality rate, especially in young adults and children. [8] This information resulted in the decision to vaccinate people in the Northern hemisphere to protect them against this severe disease. However, in the Northern hemisphere the virus behaved more like a seasonal influenza virus. H1N1 disease had the highest attack rate in young children causing relatively mild disease.[9] The pandemic H1N1 replaced the seasonal flu so that, in the influenza season 2009e2010, over 99% of the influenza positive isolates in Europe and America were pandemic H1N1 influenza A.[10,11]

There are three types of influenza viruses: types A, B and C, of which influenza A is the most virulent and causes more severe disease. Influenza A predominantly infects (aquatic) birds where extensive reassortment of genes can occur increasing the risk to form strains which can infect humans.[12] This has led to pandemics in the past (H1N1, Spanish Flu 1918 and Swine Flu 2009; H2N2, Asian Flu 1957; and H3N2 Hong Kong Flu 1968) and poses a continuous threat for new outbreaks. Due to the huge genetic variation of influenza A, different serotypes are known. Low cross protectiveness occurs throughout life and after vaccination.[13,14] Influenza B is mostly restricted to humans and has a slow mutation rate leading to a low genetic diversity; only one serotype is known.[13] Throughout life people acquire a certain degree of protection against influenza B, although antigenic drift of the virus prevents full protection.[15] Influenza C is less common then the other types and can infect humans, pigs and dogs. It most often causes mild disease in children and rarely outbreaks of severe disease.[16]

Currently two types of influenza vaccines are available, the trivalent influenza vaccine (TIV) and the live attenuated influenza vaccine (LAIV), both registered in the US and Europe. Both vaccines contain two type A influenza strains (H1N1 and H3N2) and one type B influenza strain.[17,18] Due to the high mutation rate of the virus, the vaccine composition has to be adjusted every year based on the prediction from the World Health Organization about which strains of the virus are most likely to be circulating in

the next year.[19] Both vaccines have been shown to be effective and are used in many national immunization programmes (NIP), although large differences in protection have been reported.[20,21] LAIV has only been registered for vaccination of children older than 2 years and adults younger than 50 years.[22,23] Currently no vaccine for children younger than 6 months is available. Vaccination of pregnant women in 3rd trimester with TIV may protect young infants.[24] There is the tendency to advice LAIV for pandemic influenza because it induces better protection if there is no preexisting immunity in the community against these strains.[25]

Influenza in children

Influenza infections lead to symptoms including fever, cough, nasal congestion, general malaise and muscle pain, also referred to as 'the flu' (**Table 5.1**).[26] In children, the disease is often accompanied by gastrointestinal com- plaints such as vomiting and diarrhoea.[27] In comparison to other respiratory viruses, influenza infection appears to have a more severe course of disease in children over 1 year of age, in whom infection may lead to viral pneumonia or secondary bacterial super infection.[28-30] Further, influenza infection is associated with the development of otitis media in children.[31]

Table 5.1 Symptoms of influenza infection in children.

	H1N1 (%) [8,110-112]	**Seasonal influenza (%)** [27,28,110]
Fever	91	88
Cough	82	90
Rhinorrhea	65	83
Fatigue	63	71
Sore throat	49	42
Respiratory distress	35	35
Vomiting	22	28
Diarrhea	10	12
Convulsion	8	1

The annual incidence of seasonal influenza infection is 2-10% worldwide.[2] Attack rates are highest in young children (20-30%). Before the age of 6 years most children have been infected at least once with influenza A.[32-34] The annual burden of disease in children is difficult to establish and often underestimated. This is partly due to seasonal overlap with epidemics caused by other respiratory viruses[35] and the diverse spectrum of clinical manifestations in children.[27,36] The average annual hospitalization rate in children is 9/10,000 and higher in young infants (up to 100/10,000). The overall mortality due to influenza is very low, and is the highest in children below the age of 6 months (0.9/100,000). Pre-existing medical conditions, such as chronic diseases and the use of immunosuppression, increases the risk of a complicated influenza infection.[27]

Most children with an uncomplicated influenza infection are seen by the general practitioner, demanding high healthcare capacity during the respiratory season.[37-39] Further, children with flu have to stay at home resulting in absence at work of their parents. In this way influenza infection in children also affects the community indirectly. Influenza is rapidly disseminated in school children and children play an important role in transmission cycle during an epidemic.[40,41] Vaccination of children therefore also prevents morbidity and mortality in the elderly.[42]

The Committee on Infectious Diseases of the American Academy of Pediatrics (AAP) publishes yearly the recommendations for the prevention and treatment of influenza in children.[43] Regarding vaccination, they recommend annual TIV for: (i) All children, both healthy and with conditions that increase the risk of complications from flu, aged 6 months to 18 years, (ii) household contacts and out-of-home care providers of children with conditions that place them at high risk or healthy children younger than 5 years, (iii) all healthcare professionals and (iv) pregnant women.

According to these recommendations, 85% of the US population should be vaccinated.[44] In practise, only 50% of high risk patients are vaccinated and 27% of all children in the US are completely vaccinated. Most of the children between the ages of 2 and 18 years are vaccinated with TIV (90%) and 10% with LAIV.[45] In previous recommendations of the AAP only the youngest children should be vaccinated (TIV for children 6-23 months in 2004 and 6-59 months in 2006).[46] The evidence base to vaccinate older children up to 18 years is poor.[47]

The European Centre of Disease Prevention and Control (ECDC) does not recommend routine seasonal flu vaccination in healthy children.[18,48] Since 2007 TIV has been included in the NIP of Finland for children 6-35 months.[49] During the 2009 H1N1 pandemic, many countries in Europe decided to vaccinate young children, pregnant women, the elderly and high-risk patient groups (Figure 5.1).[29] In The Netherlands, the policies regarding routine vaccination of the elderly led in 2012 to media commotion initiated by an article in the *Lancet Infectious Diseases* by Osterholm et al. and opinion articles by a general practitioner based on this article in the *Geneesmiddelen Bulletin*.[50,51] As a result of this commotion, uptake of influenza vaccines by the elderly, hospital employees and high-risk patient groups decreased, policy makers were taken to court and public concern about vaccination grew. The main arguments against vaccination were: (i) there are no randomized controlled trials with appropriate end-points in many high risk groups, (ii) there is no valid evidence from randomized studies that annual vaccination in the elderly and high risk patients is effective and (iii) 15 of the 36 studies on the effectiveness and efficacy of vaccination are sponsored by the pharmaceutical industry which raises issues of conflict of interest.[52]

Evaluation of influenza vaccinations against the Dutch criteria

In the second part of this review the current available data for influenza vaccination in children will be reviewed. The criteria to include vaccines in the Dutch NIP are used as a framework to evaluate whether influenza vaccination is justified in children (**Table 5.2**).[53]

Because data sometimes can be interpreted in different ways and new relevant studies on this subject are published every year, this review should be used as guidance to follow the ongoing discussion whether influenza vaccinations for all children should be implemented into the NIP as advised by the AAP. We will only refer to studies with laboratory-confirmed influenza infection (RT-PCR or culture) to avoid an overestimation as seen in studies only using serology.[54]

Table 5.2 Criteria used in the Netherlands to include a vaccine in the National Immunization Programme.[53]

I	The infectious disease causes considerable disease burden within the population, is serious for individuals and affects or has the potential to affect a large number of people
II	Vaccination may be expected to considerably reduce the disease burden within the population
III	Any adverse effects associated with vaccination are not sufficient to substantially diminish the public health benefit
IV	The inconvenience or discomfort that an individual may be expected to experience in connection with his personal vaccination is not disproportionate in relation to the health benefit for the individual concerned and the population as a whole
V	The balance between the costs of vaccination and the associated health benefit compares favorably to that associated with other means of reducing the relevant disease burden
VI	Relative to other vaccinations that might also be selected for inclusion, provision of this vaccination serves an urgent public health need at reasonable social cost

Disease burden and mortality of influenza in children

For children with influenza, medical attention is often sought at the general practitioner and outpatient clinics. Consultation rates vary between seasons and are age dependent. In general, children younger than 2 years are most frequently seen by GPs, with rates up to 1000/10,000 in seasons with a high incidence of influenza.[27,38,39] Between the ages of 2-5 years, the GP visit rates decline to 30-150/10,000. Data for children older than 5 years with laboratory confirmed influenza are not available, but it is likely that a further decline is seen in this age group.[27]

Frequently children are hospitalized due to influenza infection and this contributes considerably to the (limited) admission capacity of the pediatric wards. Hospitalization rates for children younger than 5 years are reported between 4 and 100/10,000 depending on the season.[27,28,37-39,55-60] Hospitalization rates due to influenza type B are lower.[56] Hospitalization rates of young children, in particularly below the age of 6 months, are the highest.[27,55,57,58,60] Hospitalization rates of children between the ages 5-16 years are relatively low and between 0.5 and 4/10,000.[55,58,59]

Mortality due to influenza infection in children is rare in developed countries. Incidences have been reported up to 0.9/100.000.[61] In the US (313.7 million people), 166 children died between 2004 and 2007 due to influenza infection. Only 6% of them were fully vaccinated. Bacterial co- infection was confirmed in 6-34% of these cases.[62] Although mortality due to influenza is rare, young children below the age of 6 months are at highest risk.

Efficacy and effectiveness

The performance of a vaccine is assessed by its efficacy and effectiveness. Vaccine efficacy is defined as the relative reduction in influenza risk given ideal circumstances and 100% vaccine uptake. This is usually determined by a randomized placebo-controlled clinical trial. Vaccine effectiveness is defined as the relative reduction in influenza risk when a vaccine is used in routine circumstances in the community. This is measured in an observational studies that use medically attended, laboratory-confirmed influenza as primary outcome.[63] Based on these definitions a meta-analysis was performed by Osterholm et al. to evaluate influenza vaccination in adults and children.[50] In this analysis, 9 randomized controlled trials were included that evaluated

Figure 5.1 Countries that have introduced national influenza A (H1N1) vaccination programmes.

vaccine efficacy in children: 1 studied TIV and 8 studied LAIV. There was no efficacy of non- adjuvanted TIV in children aged between 6 and 23 months.[64] For children aged between 2 and 18 years, no randomized controlled trials have been performed to date. LAIV vaccination in children between 6 months and 7 years was efficacious in all 8 studies, although there was a considerable variation between the studies (57-93%).[65-70] The variation in efficacy of LAIV cannot be explained by differences in study design and is most likely attributed by seasonal variability and age groups included in the studies. No randomized studies have been performed to study the efficacy of LAIV in people aged between 8 and 18 years. Further, it should be noted that the incidence of influenza in a specific season is variable and unpredictable, therefore reducing the precision of vaccine efficacy measures during the mild seasons.[71]
The meta-analysis for effectiveness of vaccination for seasonal flu with LIV showed a large variation with overall moderate protection for children (10-84%).[72-76] One study reported a high effectiveness of TIV with relative risk reduction for influenza A up to 100% for children aged between 2 and 3 years.[75] However, this was only shown during one season and as stated before, seasonal variation can have a major impact on these measurements. In two other studies it was shown that adjuvants increased the effectiveness of TIV in children aged between 6 and 72 months. A risk reduction was reached of 79% for children aged between 6 and 36 months (40% without adjuvants) and 92% for children aged between 36 and 72 months (45% without adjuvants).[77,78]
Overall it can be concluded that vaccination may lead to a considerable reduction of disease burden. However, efficacy in young infants is suboptimal and for those with the highest risk (younger than 6 months) no vaccine is approved. Furthermore, it should be noted that annual antigenic match for A/H3N2 subtypes (1991e2007) is relatively low (55-69%) and for B subtypes highly variable (38-100%).[79] This will have major impact on the eventual effectiveness of vaccination. Therefore it is questionable if there is sufficient evidence and efficacy for inclusion of influenza vaccination in a NIP.

Adverse effects

Vaccination with TIV is associated with mild local reactions such as soreness or tenderness at the injection site. Fewer than 1% of adults immunized will also experience fever, chills, or a general sense of feeling unwell that lasts one to two days. These local reactions are not different between adjuvanted and non-adjuvanted TIV. Children are more likely to experience these symptoms. During the 2009 H1N1 pandemic influenza, more than 90 million people were vaccinated with non-adjuvanted TIV and adverse events have been monitored extensively due to public concerns regarding the safety of the vaccine. There were no adverse events associated with the use of this vaccine in children.[80] The safety of the adjuvanted TIV (MF59) is comparable with non-adjuvanted vaccines in children.[81,82] Increased incidence of Guillain-Barre´ has been reported for AS03 adjuvanted vaccines, but has been refuted in large observation studies.[83-85] Narcolepsy has been associated with the AS03 adjuvanted pandemic H1N1 vaccine in children and appears to be irreversible.[86-89]

Vaccination with LAIV is associated with mild local symptoms such as rhinorrhea and nasopharyngeal congestion. Initially it was feared that LAIV in young children was associated with Bell's palsy. However, this has not been observed in large cohort studies.[90,91] It has been reported that LAIV is associated with wheezing in young children under the age of 2 years, although the incidence was not higher compared to TIV.[92,93] Only mild symptoms of rhinorrhea and headache are consistent in all studies.[94]
In animal models it has been shown that infection by seasonal flu induces hetero-subtypic protection (beneficial), whereas vaccination of mice and ferrets provides less protection against H5N1 compared to prior infection with seasonal flu.[95,96] This could imply that vaccination reduces 'natural' protection against subsequent influenza infections. In contrast, for LAIV it has been demonstrated that CD4þCD8þ and gamma/delta T-cells are induced which are relevant for hetero-subtypic immunity.[97-100] Whether these immunological mechanisms play a relevant role on a population level and should be taken in account for vaccination policies needs to be further explored.[101]
In general it can be concluded that non-adjuvanted TIV and LAIV are only associated with mild adverse events. For children below the age of 2 years, data is limited and more studies are needed.

Number needed to vaccinate

The number needed to vaccinate to reduce overall disease burden can be expressed by prevention of outpatient visits, hospitalization or mortality. Lewis et al. included three studies for a meta-analysis and based on these studies it was calculated that 12-42 children aged between 6 and 59 months need to be vaccinated to prevent one outpatient visit.[102] To prevent one hospitalization, 1031-3050 children aged between 6 and 23 months and 4255-6897 children aged between 24 and 59 months need to be vaccinated.[102] No data are available regarding the number needed to vaccinate to prevent one death, due to the low mortality rates. These numbers indicate that an enormous number of children need to be vaccinated to reduce the burden of disease. It can be argued that vaccination of children may also prevent disease burden in the elderly by herd immunity and therefore the reduction of disease burden is higher.[42] This was observed during the introduction of vaccination of school children in Japan and the US where morbidity and mortality rates due to influenza in the elderly decreased substantially.[103,104] However, it is questionable whether it is ethical to vaccination children for this purpose.

Cost effectiveness

Prevention of influenza in children affects the consultation rate of healthcare workers and has an impact on the community, in terms of absence from school and parental work. This will directly result in cost reduction for healthcare providers as well as the community. In a sensitivity analysis from the healthcare provider perspective in Finland, it was calculated that vaccination was cost saving for all age groups. This was particularly evident in children aged 6-26 months.[105] In this model, they used a moderate efficacy figure of 60% in account, which is feasible with current vaccines. These results

are comparable with calculations based on the situation in the US and the UK.[106-108] The largest reduction cost (>50%) was achieved by prevention of otitis media in children younger than 3 years of age. In these models, the effect of childhood vaccination on the incidence of influenza in the elderly was not included and indirect cost savings might be much higher. These data indicate that vaccination of healthy children is cost effective.

In relation to other vaccines

The first five criteria discussed in this review assess objective measures of vaccine efficacy, effectiveness and necessity such as burden of disease, quality adjusted life years (QUALYs) and cost-effectiveness ratio. These criteria can be compared and guide healthcare policy regarding vaccination.[109] However, both the financial resources and practical scope for including new vaccinations in the NIP are finite. Therefore priority should be given to vaccinations that serves the most urgent public health need, as measured by greatest public health benefit at reasonable individual and societal costs.[53] This leads to a more subjective criterion: what is the most urgent or potentially most urgent public health need? No good criteria are currently available to compare vaccine candidates based on their urgency. As a result, in the final decision to include vaccines in NIP, politics are an important factor and public debate inevitable.

Conclusions

When the criteria of Houweling et al. are used to evaluate whether influenza vaccination of children should be implemented in the NIP, it is evident that not all criteria are met. Although currently safe and effective vaccines for children are available, they have not been tested in children under the age of 6 months, in which the burden of disease is the highest. Further, the number needed to vaccinate is enormous to prevent significant healthcare use and cost. Importantly, to have a significant benefit on a population level, high vaccination coverage is needed and therefore public support is mandatory.
The community can be critical on the subject of vaccination and discussions via internet and social media have a major impact. To maintain public support and high vaccination coverage in the NIP, inclusion of new vaccines should be based on scientific evidence. Based on the current-available literature, we conclude that sufficient evidence for universal influenza vaccination of healthy children is lacking.

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Part II

Respiratory syncytial virus

6

Assessing severity of disease in children with respiratory viral infections

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Submitted

Abstract

Introduction: Respiratory viruses causing lower respiratory tract infections (LRTI's) are a major cause of hospital admissions in children. Since the course of these infections is unpredictable with potential fast deterioration into respiratory failure, infants are easily admitted to the hospital for observation. The aim of this study was to examine whether systemic inflammatory markers can be used to predict severity of disease in children with respiratory viral infections.

Methods: Blood and nasopharyngeal washings from children < 3 years of age with viral LRTI attending a hospital were collected within 24 hours (acute) and after 4-6 weeks (recovery). Patients were assigned to a mild (observation only), moderate (supplemental oxygen and/or nasogastric feeding) or severe (mechanically ventilation) group. Linear regression analysis was used to design a prediction rule using plasma levels of CRP, SAA, PTX3, SAP and properdin. This rule was tested in a validation cohort.

Results: One hundred and four children (52% male) were included. A combination of CRP, SAA, PTX3 and properdin was a better indicator of severe disease compared to any of the individual makers and age (69% sensitivity, 90% specificity). Validation in 141 patients resulted in 71% sensitivity, 87% specificity, NPV 64% and PPV of 90%. The prediction rule was not able to identify patients with a mild course of disease.

Conclusion: A combination of CRP, SAA, PTX3 and properdin accurately identifies a severe course of disease, especially in children under two months of age.

Strengths and limitations of this study

- Identification and validation of novel markers to assess severity of disease in children with viral LRTI
- Combining biomarkers in linear regression model to optimize performance
- Biomarkers and regression model adjusted for children under 2 months of age
- Other promising inflammatory markers, such as procalcitonin, were not included and could have further increased the clinical performance of test.
- Study performed during admission hospital and ICU, validation needed in earlier stages of disease at ER and GP-office

Introduction

Acute lower respiratory viral infections (LRTIs) annually result in hospitalisation of 0.9-1.36% of a birth cohort.[1,2] Eventually, 6 to 15% of these patients are admitted to the intensive care unit.[1,3] Young age and prematurity are the most important risk factors for severe disease. This is reflected by the fact that over 50% of the children admitted to the ICU have no other risk factors besides young age.[1,4,5]

Infants under 2 to 3 months of age are easily admitted to the hospital due to a potentially rapid progression of disease. More than one third of all patients hospitalized with acute respiratory viral infections do not receive supportive care.[5] However, 5% of the patients discharged from the emergency room require readmission at a later stage of the infection.[6] Therefore, there is a need for biomarkers that can predict the course of disease in infants with viral respiratory tract infections.

Currently, there are clinical prediction rules to predict safe discharge, length of hospitalisation and ICU admission for children with acute respiratory viral infections based on demographic criteria and clinical symptoms.[4,5,7-11] Although these clinical prediction rules show the potential to improve clinical judgement, validation in other cohorts is often lacking or implementation in daily practice is difficult due to subjective criteria.[8,9] Moreover, children under two months of age were either excluded or the studies were designed in such a way that all patients in this age group were automatically admitted to the hospital. A more objective, reproducible and sensitive prediction of disease severity for all age groups may be achieved by using biochemical and hematological markers.[12-16]

Therefore, plasma proteins that were not yet extensively studied in the context of viral LRTI were identified from literature. Pentraxin 3 (PTX3) has been described as an early sensitive and specific marker for bacterial pneumonia in broncho-alveolar lavages of mechanically ventilated adults, and as a severity marker of meningococcal disease in children.[17,18] In 2016 a study was published that showed that PTX3 levels are also a marker for LRTI in children and is more sensitive than CRP, which is frequently used in clinical care.[19] No correlation between CRP levels and severity of viral disease has been described. When CRP levels are very high they indicate bacterial infection, however often CRP is moderately increased and then CRP is not able to differentiate between viral or bacterial aetiology of infection.[20-23] *Serum amyloid A* (SAA) appears to be more sensitive than CRP for the detection of bacterial infections in neonates, although it is not used in daily practice.[24] The SAA/CRP ratio can be used as a marker for severe disease in children with bacterial infections.[25] Nakayama *et al.* have shown that SAA levels increase in children with different viral infections, including viral LRTI. However, an association with disease severity was not established.[26] *Serum amyloid P component* (SAP) can activate the classical complement pathway and interacts with mannan-binding lectin (MBL). SAP is involved in inflammation and used as a marker for atherosclerosis and auto-immune disease.[27] Skinner *et al.* have shown that SAP levels were not increased during bacterial pneumonia, suggesting a limited potential as biomarker in

infectious disease.[28] No studies were found that assessed the role of SAP during viral infections.

Properdin (CFP) is a positive regulator of the alternative pathway of the complement system and it is a pattern recognition molecule that can bind to apoptotic/necrotic cells or microbial pathogens (including viruses) to facilitate phagocytosis and clearance.[29,30] Familial deficiencies in CFP are known and are associated with susceptibility to meningococcal infections.[31,32] However, an increased susceptibility to viral infections has never been published.

In this study we tested the aforementioned plasma proteins for their correlation with severity of viral lower respiratory tract infections in children.

Materials and Methods

Study design

Children younger than 3 years of age were eligible for inclusion if they attended one of the two hospitals in Nijmegen from September until May with symptoms of an acute viral lower respiratory tract infection (LRTI). Symptoms included signs of increased respiratory effort (e.g. tachypnea and/or use of accessory respiratory muscles or retractions) and/or expiratory wheezing and/or crackles and/or apnea. Patients with congenital or acquired immune deficiencies, immunosuppressive medication (including $>$ 24 hours of corticosteroids) or severe psychomotor retardation were excluded. Eligible patients were identified by their physician, who notified the study team. When informed consent of both parents was obtained within 24 hours of admission patients were included in the study. Data on clinical parameters, course of disease, medical history and demographics were retrieved from medical records and questionnaires. Retrospectively, after discharge patients were allocated into three groups: mild, send home or only clinical observation; moderate, patients with need for supplemental oxygen and/or nasogastric feeding; and severe, patients with mechanical ventilation. Supplemental oxygen was started according to the protocols from the clinical wards in patients with an oxygen saturation of $\leq$ 92% for at least 10 minutes after use of decongestives. Results were evaluated in a training cohort (n=104, 2010-2012) and subsequently tested in a larger historical validation cohort with the same inclusion and exclusion criteria (n=141, 2006-2009). The study was approved by the Committee on Research involving Human Subjects.

Material collection

After informed consent, blood and a nasopharyngeal wash were collected within 24 hours (acute), and after 4-6 weeks (recovery). A venous blood sample (3ml) was collected in sodium heparin tubes and immediately transported to the laboratory. Plasma (and cell fractions) were obtained after density centrifugation (Lymphoprep®, Axis Shield, Norway) and stored at -80°C. After instilling 0.5 ml of saline into one of the

nostrils, a catheter was introduced into the nasopharynx to aspirate nasopharyngeal fluid. Samples were instantly put on ice, transported to the laboratory and stored at -80°C. Multiplex RT-PCR was performed on the nasal wash to confirm viral aetiology of disease, as previously described.[33] The assay was designed to detect fifteen viruses.

ELISA

Levels of SAA, SAP, PTX3, CFP and CRP were determined in two fold diluted plasma by ELISA following the manufacturer's instructions (Hycult Biotech, Uden, The Netherlands, Catalog No's HK333, HK331, HK347, HK334 and HK358, respectively)

Statistics

Data are expressed as percentages for categorical variables and as mean and standard error of the mean (SEM) or median and inter quartile range (IQR) for the non-parametric continuous variables. Kruskal-Wallis tests were performed on continuous variables, followed by Mann-Whitney U to further analyze differences. For categorical data, Chi-square testing was used. Paired data was tested with the Wilcoxon Signed-Rank Test. Correlations were calculated using Spearman's rho. The optimal predictive combination of proteins was selected using the M5 method linear regression, which removes classifiers with the smallest standardized coefficient one-by-one until no decrease in error estimate is observed . The outcome of the obtained rule was analysed for its Receiver Operator Characteristics (ROC). Cut-off values were determined for both mild and severe disease. These cut offs were subsequently validated in a second cohort. A two-sided p value of < 0.05 was considered statistically significant. Statistical tests were performed by SPSS for Windows (Release 19, SPSS Inc., Chicago, IL) and Waikato Environment for Knowledge Analysis (WEKA) was used to perform linear regression. [34]

Results

Patient characteristics

The training cohort consisted of 104 children (overall inclusion percentage 60%) (**Table 6.1**). Children with severe disease were significantly younger and had more often siblings than patients in the mild and moderate groups. Duration of hospitalisation significantly increased towards more severe disease. 2% of the patients were not hospitalised (2/104), whereas 17% of the hospitalised patients had only mild disease. RSV was detected in the majority of patients (65%), in 43% viral co-infections were present. The highest proportion of RSV mono-infections was seen in children with a severe course of disease ($p<0.001$), as was previously published by our group.[33]

Table 6.1 Patient characteristics.

	Total N=104 (%)	Mild n= 20 (%)	Moderate n=52 (%)	Severe n=32 (%)	p-value
Gender (male)	54 (52%)	12 (60%)	24 (46%)	18 (56%)	NS
Age (days)	92 [38.2-289.8]	129 [63.0-368.3]	165.0 [66.0-422.0]	37.5 [22.0-86.5]	<0.001*
Prematurity (< 35 wks)	5 (5%)	1 (5%)	3 (6%)	1 (3%)	NS
Onset symptoms (days)	3.0 [2.0-4.8]	2.5 [1.3-3.8]	3.0 [2.0-5.0]	4.0 [2.0-4.8]	NS
Duration admission (days)	6.5 [3.3-10.8]	2.0 [2.0-3.0]	6.0 [4.0-8.0]	11.0 [9.3-13.8]	<0.001**
Supplemental oxygen	78 (75%)	0	46 (89%)	32 (100%)	<0.001***
RSV infection	68 (65%)	8 (40%)	38 (73%)	23 (72%)	<0.05 ****
% mono infection RSV	39 (57%)	3 (37,5%)	20 (53%)	16 (70%)	<0.001

Values are given in numbers (percentages) or medians [25th – 75th percentiles]. * mild vs severe p=0.001, moderate vs severe p<0.0001 ** mild vs moderate/severe p<0.0001, moderate vs severe p<0,0001 *** mild vs moderate/severe (p<0,0001), moderate vs severe p<0,05 **** mild vs moderate/severe (p<0.05).

Markers for infection and disease severity

Plasma levels of CRP, SAP, SAA and PTX3 increased significantly during disease compared to levels in recovery samples whereas CFP levels did not differ between these two time points (**Figure 6.1A-B**). When the acute disease group was subdivided into three severity classes, plasma levels of CRP during severe disease were significantly higher compared to patients with mild and moderate disease (**Figure 6.2A**). Although there was a stepwise incline in plasma levels of PTX3, there were no significant differences (**Figure 6.2B**). Plasma levels of SAP were also equal between the different disease severities (**Figure 6.2C**). SAA plasma levels however, were significantly higher during severe disease compared to mild disease (**Figure 6.2D**). CFP plasma levels did not differ between mild and moderate disease. In contrast to the other markers, the CFP plasma levels dropped significantly in patients with severe disease (**Figure 6.2E**).
Since disease severity can also be reflected by the duration of hospitalisation and the need for supplemental oxygen (as substitute for the need for hospitalisation) we tested whether they correlated with the plasma proteins. Both CRP and CFP correlated significantly with duration of hospitalisation ($\rho=0.361$, $p\leq0.001$ and $\rho=-0.22$, $p<0.05$, respectively). However, PTX3, SAP and SAA levels did not. The need for supplemental oxygen was associated with higher levels of CRP and PTX3 (**Figure 6.2A-B**), whereas the other markers showed no significant difference.

The influence of age and viral aetiology on protein levels

Children with severe disease are significantly younger (**Table 6.1**), therefore we checked whether age is a confounder for our protein levels. After stratification of the cohort per severity and age group (under and above two months of age) protein levels were

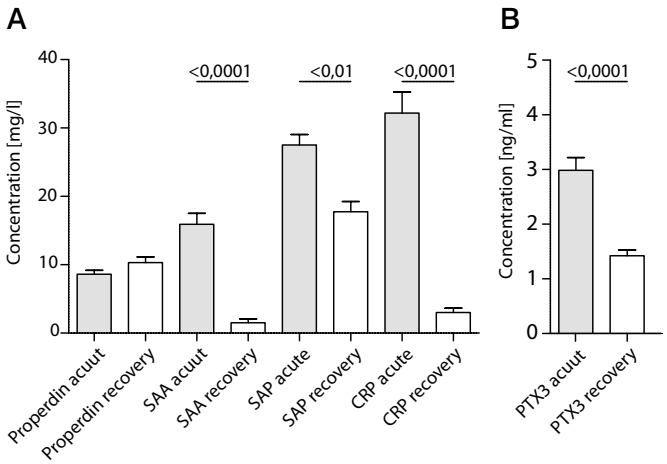

Figure 6.1 Comparison of plasma protein levels in children with acute respiratory viral infections (n=104) with their paired recovery sample (n=51) taken 4-6 weeks after acute infection are shown for CFP, SAA, SAP, CRP (A) and PTX3 (B) (Wilcoxon matched-pairs signed rank test).

compared. After Bonferroni correction no significant differences were found in the group under two months of age. Thus, age is no confounder for our markers in the prediction of severe disease.

RSV mono-infections are more frequently seen in the severe disease group (**Table 6.1**). We tested whether patients with RSV mono-infections also had higher plasma levels compared to patients with the same disease severity without RSV mono-infections. We found that patients with RSV mono-infections had significantly higher SAA levels during severe disease ($p<0.05$). Levels of all other inflammatory markers were equal.

Accuracy of the inflammatory markers to predict disease severity

After finding differences in plasma levels in patients with different disease severities the diagnostic performance of the proteins was tested. First, the performance of the markers to indicate the need for hospitalisation (supplemental oxygen) was assessed. Of 104 patients included for analysis 27 did not receive supplemental oxygen: 6 of these patients belonged to the moderate group (**Table 6.1**). The area under the curve (AUC) for the markers CRP and PTX3 was 0.63 and 0.64 respectively. The AUC for the other markers was 0.59 for SAA, 0.57 for SAP and 0.51 for CFP.

Second, the performance of markers to indicate the need for mechanical ventilation (severe disease) was assessed. Of 104 patients included for analysis, 32 were mechanically ventilated (**Table 6.1**). The AUC of the markers that were associated with severe disease, CRP, SAA and CFP, was 0.73, 0.57 and 0.76 respectively. The AUC for the other markers was 0.56 for PTX3 and 0.53 for SAP.

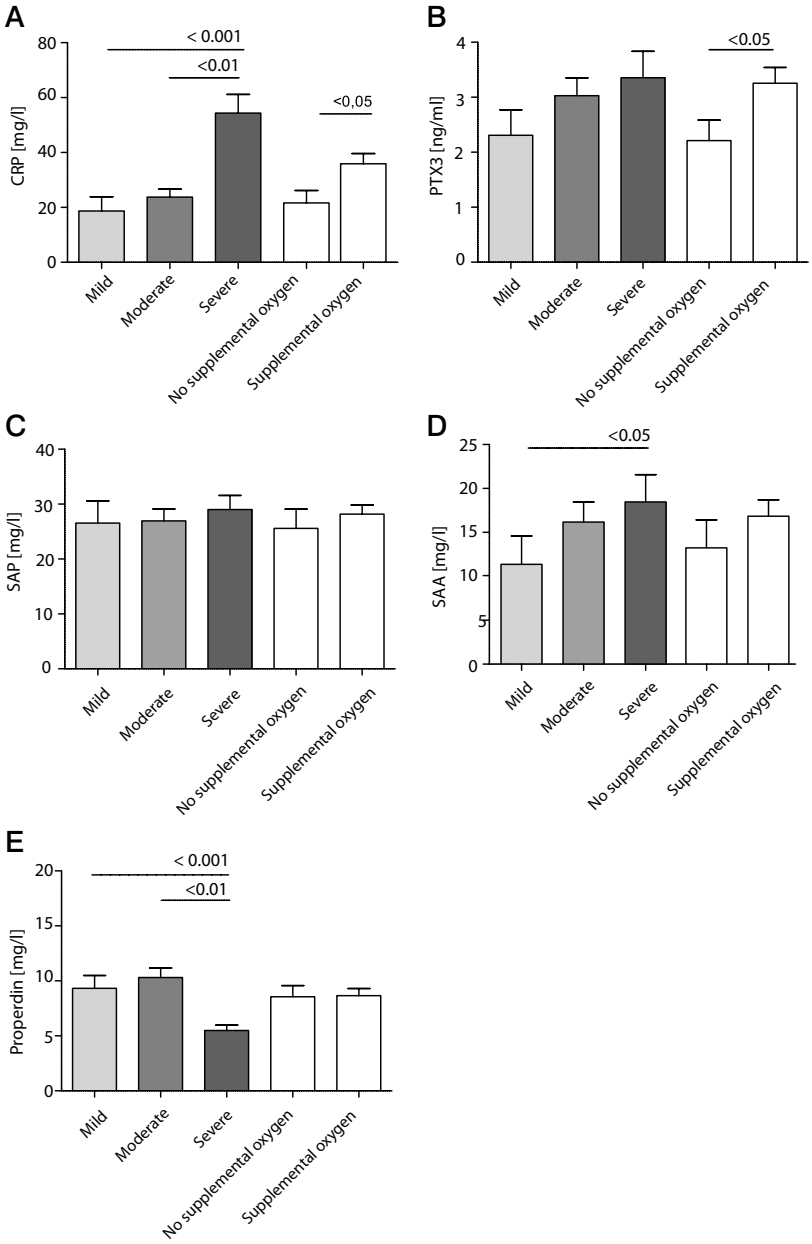

Figure 6.2 Comparison of protein levels between patients with mild (n=21), moderate (n=51) and severe (n=32) disease and for patients with (n=77) and without (n=27) the need for supplemental oxygen are shown for CRP (A), PTX3 (B), SAP (C), SAA (D) and CFP (E) (Mann Whitney U test).

Combining inflammatory markers increases performance

As reflected by the AUC, none of the single markers was considered sufficient to identify mild disease and thus aid in the decision to discharge a patient. Whereas the AUC of some markers for severe disease were slightly higher, none of them exceeded 0.8.
Therefore, linear regression modelling was used to analyse whether a combination of markers could increase the individual performance. The assumption was that distances between the three groups were equal. The linear regression resulted in the following model: (5.8 x PTX3) + (1.15 x CRP) – (4.1 x CFP) – (0.93 x SAA) + 106.25. The AUC for this rule is 0.65 for mild disease (no need for supplemental oxygen) and 0.89 for severe disease (mechanical ventilation).
Based on the ROCs, a cut-off value for the identification of mild disease was set at 93.50, which resulted in an AUC of 0.65 and sensitivity of 55%, a specificity of 70%, a positive predictive value (PPV) of 39% and a negative predictive value (NPV) of 82%). In $<$ 2 months old children the AUC is 0.88 (sensitivity 87%, specificity 71%, PPV 93% and NPV 56%), whereas in $>$ 2 months old patients the AUC is 0.50 (sensitivity 57%, specificity 47%, PPV 72% and NPV 31%).
The cut-off value for severe disease was set at 122 (sensitivity 69%, specificity 90% with a PPV of 76% and an NPV of 87%) with an AUC of 0.88. In children below two months of age the AUC for severe disease increased to 0.90 (sensitivity 72%, specificity 85%, PPV 82% and NPV 77%). In older children the AUC is 0.86 (sensitivity 55%, specificity 93%, PPV 60% and NPV 91%).

Validation of prediction rules

A new cohort of 141 children was tested. Patient characteristics of the original and validation cohort were similar with respect to their distribution of age, gender and number of prematurely born infants (less than 35 weeks). In contrast to the training cohort, the presence of siblings was not significantly different between the severity groups in the validation cohort (**Supplemental Table 6.1 and Supplemental Figure 6.1**).

Need for supplemental oxygen

Validation of the prediction rule for mild disease, need for hospitalization, resulted in 51% sensitivity and 68% specificity (PPV 46% and NPV 72%). In children below two months of age the rule performed better the AUC increased from 0.67 to 0.79 (sensitivity 55%, specificity 81%, PPV 46% and NPV 86%). In children $>$ 2 months the AUC decreased to 0.58 (sensitivity 52%, specificity 60%, PPV 42% and NPV 69%).

Need for mechanical ventilation

When the prediction rule was applied to the validation set with a cut-off of 1.22, the sensitivity for severe disease was 71% with 87% specificity, PPV and NPV were 64% and 90%, respectively.

In children below two months of age the AUC for severe disease is 0.87 (sensitivity 77%, specificity 57%, PPV 45%, NPV 84%). In older children the AUC remains 0.87 (sensitivity 58%, specificity 90%, PPV 52%, NPV 92%).

Discussion

In this study, we show that plasma levels of CRP, PTX3, SAA and CFP correlate with disease severity in children with acute viral LRTI. Furthermore, we demonstrate that a combination of these markers significantly increased the performance of the individual proteins to identify patients with severe disease. The designed prediction rule has been validated in a larger independent patient cohort with similar characteristics, which resulted in a comparable performance. Moreover, the performance of the rule was best in patients under two months of age (<60.5 days) in whom it is clinically difficult to predict the course of disease.

Interestingly, most of the inflammatory markers investigated in this study have not been described in the context of viral LRTI or disease severity. The increase of CRP and SAA levels during viral infection was known and this was also the reason to include the proteins in our study. We were able to confirm the work of Huttunen *et al.* that the ratio SAA/CRP is significantly decreased in patients with severe disease compared to patients with mild and moderate disease (data not shown).[25] Moreover, CRP and SAA levels both correlated with severity of viral LRTI's and CRP was also correlated with length of hospitalisation. To the best of our knowledge, these findings have not been described before. The increase in PTX3 levels during more severe disease and a correlation with need for supplemental oxygen show the potential of PTX3 as biomarker for viral LRTI in children. This was already proposed by others, who saw a correlation with peak temperature, duration of fever and presence of pneumonia in mechanically ventilated patients.[17,19] Although SAP levels significantly increase during infection, no correlation could be observed with disease severity in children with viral LRTI. Therefore, it appears that SAP could not be used as a biomarker for disease severity in viral or bacterial infections.[28] This study is the first to describe the remarkable kinetics of CFP (Properdin) levels, which decrease during severe viral infection in children. We were unable to find an explanation in literature. However, since CFP levels restored in the available recovery samples, a deficiency is highly unlikely. Other possible explanations for the decrease might be consumption, as occurs with other complement factors, or an (unrecognised) bacterial co-infection. Although cultures were routinely taken at the intensive care unit before the start of SDD antibiotics, bacterial infections cannot be ruled out completely. A sub analysis of patients with (n=10) and without (n=15) positive bacterial cultures on the ICU showed no difference in protein levels (data not shown). During the study period selective digestive decontamination (SDD) was standard practice in the ICU and therefore all severe patients would have had at least one gift of prophylactic antibiotics before the study samples were taken.

The prediction rule for the identification of children with a mild course of disease appeared to be insufficient for clinical use (AUC 0.65). However, the performance in children under two months of age (AUC 0.88) is promising and might provide additive value in clinical decision making. Currently, there are no comparable laboratory based studies assessing the identification of mild disease. Clinical rules for safe discharge based on age and findings at physical examination have been developed. Unfortunately, in these studies children below 2 months of age were excluded or automatically admitted regardless of the necessity of treatment.[5,31,35,36]

The identification of patients with severe disease was accurate with the chosen inflammatory markers (AUC 0.89). The prediction performed better than age on itself (AUC 0.77). A prediction rule based on IL-8 and RANTES in plasma and $CD4^+$ T cell counts accurately identifies severe disease, but needs to be validated in an independent cohort to establish clinical potential.[16] A combination of biochemical, hematological and clinical data might further improve accuracy. A drawback of introducing clinical variables is that most of the published risk factors in clinical prediction are not objective or may change rapidly in time. Moreover, signs and symptoms of children can be ambiguous at the moment of presentation and only become clear after prolonged observation. Prediction rules using clinical data, often show a lower AUC and validation studies of these rules are lacking or have a smaller sample size.[4,37]

The advantage of measuring markers in plasma is the easy implementation, speed, reproducibility and standardisation. Moreover, with the use of linear regression tools it is easy to add new markers to optimize the rule. It is tempting to speculate that some of the biomarkers precede clinical symptoms and could therefore be used early in the course of disease to predict severe outcome. Currently, there is no targeted therapy for most respiratory viruses and therefore there are no consequences to the early identification of patients with a severe course of disease besides optimalisation of supportive care. The identification of children with a mild course of disease has more visible consequences, as can be measured in reduced hospitalisation rates or length of stay in the hospital. To truly estimate the value of the markers a prospective study is needed, moreover it would be interesting to measure the markers earlier in the course of disease. However, even before the markers are implemented in the clinic, biomarkers could be used to stratify patients in clinical studies and as secondary end-point in intervention studies. With the current development of point-of-care tests, plasma protein levels can be available within a relevant time frame.

Acknowledgements

We would like to thank all parents and children who participated in this study. We are also thankful for the help of the staff from the participating hospitals. We thank Tefa for their Cheiron Dynamic II apparatus to enable us to perform nasal washes in a standardized matter. The Pediatric Drug Research Centre Nijmegen, especially Mariëtte Las, for excellent support during the study. Finally, we would like to thank Marloes Vissers for critically reading this manuscript.

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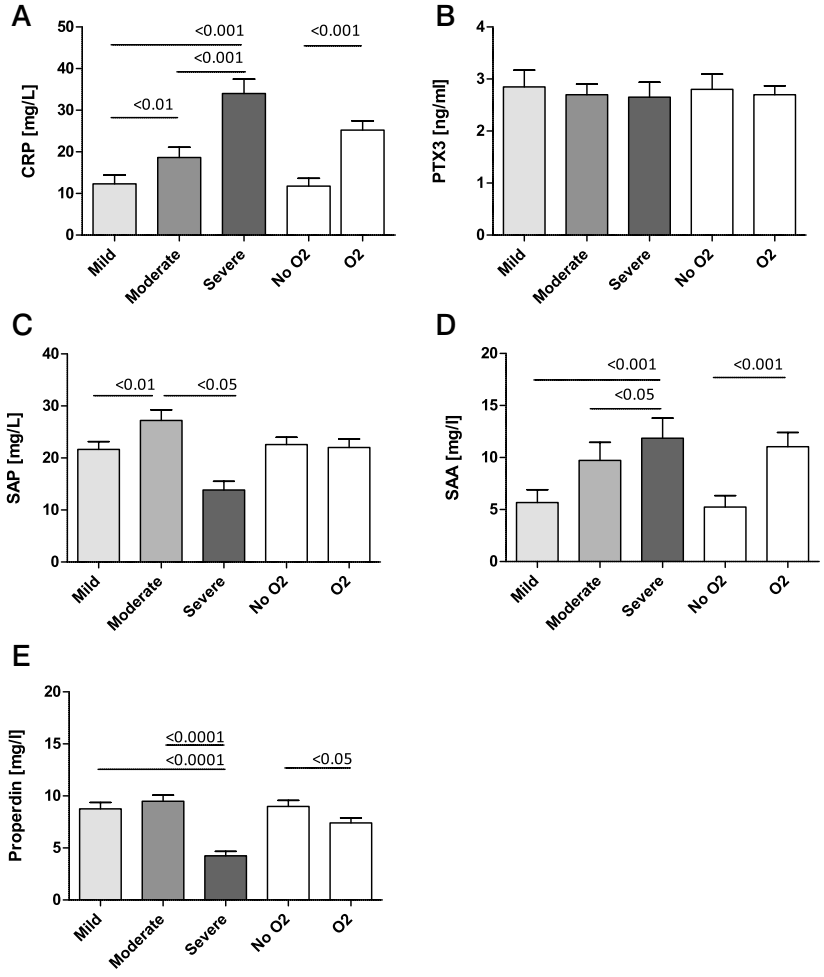
Supplemental Table 6.1 Patient characteristics validation cohort.

	Total n=141 (%)	Mild n=43 (%)	Moderate 51 n=63 (%)	Severe n=35 (%)	p-value
Gender (male)	84 (60)	24 (55)	36 (57)	24 (69)	NS
Age (days)	84 [35-211]	174 [58-299]	99 [39-220]	45 [25-96]	<0.001*
Prematurity (< 35 wks)	20 (14)	5 (12)	6 (10)	9 (26)	NS
Onset symptoms (days)	4 [3-6]	4 [3-7]	4 [3-6]	5 [3-6]	NS
Duration admission (days)	5 [2.25-8.75]	2 [0-4]	5 [3-7]	11 [8-13]	<0.001**
Supplemental oxygen	92 (65%)	0	57 (91%)	35 (100%)	<0.001***
RSV infection	94 (67)	*28 (57)*	*40 (70)*	*26 (74)*	*NS*
RSV mono-infection	60 (62.5)	*12 (24)*	*25 (44)*	*21 (60)*	*<0.05***

Values are given in numbers (percentages) or medians [25th – 75th percentiles]. *mild vs severe p<0.001 and moderate vs severe p<0.01. ** mild vs severe p<0.05. ***mild vs moderate/severe and moderate vs severe p<0.001.



Supplemental Figure 6.1 Comparison of protein levels between patients with mild (n=43), moderate (n=63) and severe (n=35) disease and for patients with (n=169) and without (n=76) the need for supplemental oxygen are shown for CRP (A), PTX3 (B), SAP (C), SAA (D) and CFP (E) (Mann Whitney U test).

7

Reduced expression of HLA-DR on monocytes during severe respiratory syncytial virus infections

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Abstract

Background: Respiratory syncytial virus (RSV) is a common cause of bronchiolitis in infants with a wide spectrum of disease severity. Besides environmental and genetic factors, it is thought that the innate immune system plays a pivotal role. The aim of this study was to investigate the expression of immune receptors on monocytes and the in vitro responsiveness from infants with severe RSV infections.

Methods: Peripheral blood mononuclear cells (PBMCs) from infants with RSV infections were isolated. Classical, intermediate and nonclassical monocytes were immunophenotyped for the expression of CD14, CD16, human leukocyte antigen (HLA)-ABC and HLA-DR. PBMCs were stimulated with lipopolysaccharide to determine the secretion of tumor necrosis factor and interleukin (IL)-10 with enzyme-linked immunosorbent assay.

Results: During RSV infection, intermediate monocytes are increased in the peripheral blood, whereas classical and nonclassical monocytes are reduced. The expression of CD14 and HLA-ABC is increased on monocytes, whereas the expression of HLA-DR is suppressed. Low HLA-DR expression is correlated with increased disease severity. PBMCs from infants with severe RSV infections show an impaired IL-10 response in vitro.

Conclusions: Phenotyping subpopulations of monocytes combined with in vitro responsiveness reveals significant differences between nonsevere and severe RSV infections. Reduced HLA-DR expression and impaired IL-10 production in vitro during severe RSV infections indicate that an imbalanced innate immune response may play an important role in disease severity.

Introduction

Respiratory syncytial virus (RSV) is a common cause of bronchiolitis in children <2 years of age.[1] The clinical manifestations of RSV infection range from a common cold to severe lower respiratory tract infections that require oxygen treatment. Although known risk factors such as prematurity, cardiopulmonary disease and immune deficiencies are used in clinical prediction models, these factors only successfully predict approximately 50% of severe RSV infections.[2] Severe RSV infections that require oxygen treatment typically occur in infants <3 months of age and usually involve primary infections. The occurrence of severe RSV infections is associated with subsequent wheezing episodes and diagnosis of asthma.[3] Tregoning and Schwarze describe several factors determining the pathogenesis of RSV disease, including physical, environmental and genetic factors.[4] In addition to these factors, it is increasingly thought that the innate immune system plays an important role in determining the clinical course of infection. Innate immune cells like monocytes are recruited from the blood stream to the site of infection, where they can either directly contribute to the host antimicrobial defense or supply the inflamed tissue with differentiated macrophages and dendritic cells to target the pathogen.[5] Circulating monocytes are part of the innate immune system and appear to be heterogenic. Different subpopulations of circulating monocytes reflect developmental stages, and each subset has a distinct role in inflammatory processes and infectious diseases.[6] Based on the expression of CD14 and CD16, monocytes can be divided into 3 subpopulations: classical $CD14^{++}CD16^{-}$, intermediate $CD14^{++}CD16^{+}$ and nonclassical $CD14^{+}CD16^{++}$ monocytes.[7,8] The regulation of the immune response and the role of monocytes and its receptors in disease severity have previously been shown for bacterial infections. The expression of human leukocyte antigen (HLA)-DR on monocytic cells is reduced during severe bacterial infections, and a possible mechanism for this correlation between low HLA-DR expression and increased disease severity is a phenomenon called immunoparalysis.[9–11] HLA-DR molecules are membrane-bound molecules that play a role in antigen presentation and T cell activation. Immunoparalysis refers to the combination of reduced HLA-DR expression and reduced in vitro responsiveness to lipopolysaccharide (LPS) resulting in severe disease progression. Although extensive research is deciphering the disease-enhancing effect of immunoparalysis in the field of bacterial infections, the role of immunoparalysis is yet to be elucidated in the context of severe viral respiratory tract infections.

In the context of RSV, the role of antiviral monocytes has been appreciated for decades and, in vitro, interaction of RSV with monocytes leads to an upregulation of innate inflammatory mediators.[12–15] These inflammatory cytokines shape the homeostatic balance for eliciting an adequate immune response. In vivo, an imbalance in cytokine production contributes to RSV pathogenesis and severe RSV infections.[16] However, it is unknown whether monocytes can play a role in the regulation of the immune response against RSV and thereby either prevent or contribute to disease severity.

In this study, we assessed immunological markers on monocytes in infants with nonsevere and severe RSV infections based on the need for oxygen treatment. For this purpose, circulating monocytes were phenotyped in a cohort of infants with RSV infections. The expression of CD14, HLA-ABC and HLA-DR was correlated with the presence of RSV infection and with disease severity. Finally, peripheral blood mononuclear cell (PBMC) stimulation was performed to assess the in vitro responsiveness of PBMCs during RSV infections in infants.

Materials and Methods

Study Design

Children <2 years of age hospitalized with RSV bronchiolitis were prospectively included from November 2010 to April 2013 after informed consent from both parents. Bronchiolitis was defined as an acute infection of the lower airways, characterized by increased respiratory effort and expiratory wheezing and/or crackles and/or apnea. Nasopharyngeal aspirates from all patients included were RSV positive. Clinical data were collected from questionnaires, including age, sex, gestational age, birth weight, breastfeeding, duration of symptoms, duration of oxygen therapy, the use of corticosteroids and/or antibiotics, vaccination with palivizumab and duration of hospitalization. Within 24 hours after presentation to the hospital, 3-mL sodium heparinized blood was collected. Retrospectively, patients were classified into 2 groups: no oxygen treatment versus oxygen treatment by nasal cannula or mechanical ventilation (when oxygen saturation was <93% for >10 minutes).[17,18] Recovery samples were collected 4–6 weeks after presentation. Samples from healthy infants undergoing elective surgery for inguinal hernia served as age- and sex-matched controls. Nasopharyngeal aspirates from all healthy controls were RSV negative.

Virus Detection

Multiplex real-time reverse transcription polymerase chain reaction for 15 viruses was performed on nasopharyngeal aspirates as previously described.[19]

Cell Differentiation

A thin blood smear of sodium heparinized blood was made, and Giemsa staining was used to enable cell differentiation.

Isolation of PBMCs

PBMCs were isolated using Lymphoprep (Axis-Shield, Oslo, Norway). Briefly, blood was anticoagulated with sodium heparin (BD Vacutainer, Becton Dickinson, Franklin Lakes, NJ) and diluted with an equal volume of phosphate-buffered saline. The diluted blood was carefully added on top of the Lymphoprep and centrifuged at 800*g*. PBMCs were harvested, washed twice in phosphate-buffered saline and counted by the use of

a hemocytometer. PBMCs from infants with RSV infection were stored in liquid nitrogen in 10% fetal calf serum and 10% dimethylsulfoxide.

Stimulation of PBMCs

Directly after collection, 5×10^5 PBMCs in a 100μL volume were added to an equal volume of Roswell Park Memorial Institute medium (negative control) or LPS (*Escherichia coli* 1 ng/mL) and incubated at 37°C and 5% CO_2. After 20 hours, the supernatant was stored at −20°C for cytokine measurement.

Immunophenotyping

PBMCs were thawed and leukocyte subsets were determined using fl w cytometry; 2×10^5 PBMC were incubated with 50μL flurochrome-labeled monoclonal antibodies for 30 minutes in the dark on ice in 96-well microtiter plates. CD14 V500, CD16 Alexa Fluor 700, HLA-ABC V450 and HLA-DR Qdot605 were used (Becton Dickinson). Results were acquired on a BD LSR II flow cytometer, and data were analyzed using FlowJO (Tree Star, Inc, Ashland, OR). For gating, live cells were selected as 7-AAD-negative cells. Next, HLA- DR-positive, HLA-ABC-positive and SSC high cells were selected to include antigen-presenting cells and exclude NK cells, T cells and B cells. CD14-positive cells were selected and divided into CD16- negative and CD16-positive cells to select classical $CD14^{++}CD16^{-}$, intermediate $CD14^{++}CD16^{+}$ and nonclassical $CD14^{+}CD16^{++}$ monocytes. CD14, HLA-ABC and HLA-DR expressions were measured by the geometric mean of fluerescense intensity.

Enzyme-linked Immunosorbent Assay

Tumor necrosis factor (TNF) and interleukin (IL)-10 concentrations were measured in supernatant of the stimulation assay with commercial enzyme-linked immunosorbent assay kits (PeliKine Compact, Sanquin, the Netherlands) according to the manufacturer's instructions. The detection limits were 78 and 9.4 pg/mL, respectively.

Statistical analysis

The distribution of categorical variables was presented as percentages per category. Expression levels of membrane-bound receptors were depicted as geometric mean fluorescence intensity and presented as medians with interquartile range. The Kruskal-Wallis test was performed followed by the Mann-Whitney *U* test for individual comparisons. Cytokine values were presented as mean with standard error of the mean, and Mann-Whitney *U* test was performed for statistical analysis. Pearson tests were performed to test for correlations. A value of $P < 0.05$ was considered statistically significant. All statistical tests were performed with GraphPad (GraphPad Inc, La Jolla, CA).

Study Approval

The study protocols were approved by the Regional Committee on Research involving Human Subjects Arnhem-Nijmegen (serving as the Institutional Review Board) and

were conducted in accordance with the principles of the Declaration of Helsinki. Written informed consent was obtained from the parents of all children.

Results

Characterization of Study Population

Forty-eight patients were included for flowcytometry analysis. Fifteen healthy controls were included for comparison. There was no significant difference in age and sex between groups (see **Table 7.1**). The gestational age and birth weight of the healthy controls were lower compared with those of RSV-infected infants. There was no difference in the presence of prematurity between the groups (see **Table 7.1**). There was no difference in gestational age between infants without oxygen treatment and infants with oxygen treatment (see **Table7. 1**). In a second cohort for in vitro responsiveness to LPS, 37 patients and 12 healthy controls were included. There was no significant difference in age, sex or prematurity between groups. The gestational age and birth weight of the healthy controls were lower compared with those of RSV-infected infants (see Table, Supplemental Digital Content 1, http://links.lww.com/INF/C336).

Total Monocyte Count Is Not Increased During Acute Infection and Does Not Discriminate Between Disease Severity

There is no significant difference in the absolute number of peripheral monocytes of infants during RSV infection compared with the recovery phase (**Figure 7.1**). There is no difference in monocyte count between disease severity groups (**Figure 7.1**).

The Percentage of Intermediate Monocytes Is Increased During RSV Infection

We first determined whether the subsets of monocytes in the peripheral blood are different during RSV infection compared with healthy controls or recovery samples. Monocytes were divided into 3 subpopulations based on the expression level of CD14 and CD16: classical $CD14^{++}CD16^{-}$, intermediate $CD14^{++}CD16^{+}$ and nonclassical $CD14^{+}CD16^{++}$ monocytes. The main population of circulating monocytes consists of classical monocytes, and the percentage of these classical monocytes was significantly lower in the acute phase compared with the recovery phase (**Figure 7. 2A**). During RSV infection, a significantly higher percentage of intermediate monocytes compared with healthy controls and with the recovery phase was observed (**Figure 7.2A**). The percentage of nonclassical monocytes is significantly lower in the acute phase compared with healthy controls and the recovery phase (**Figure 7.2A**). Besides a reduced percentage of classical monocytes in RSV-infected infants with oxygen treatment, no differences were observed between disease severity groups (**Figure 7.2B**). These data suggest that the pool of peripheral blood monocytes is dynamic and changes in favour of intermediate monocytes during RSV infection.

Table 7.1 Cohort Characteristics.

	Healthy (n= 15)	No Oxygen (n = 8)	Oxygen (n =40)	*p* Value
Age (d)	79.0 [68.0–94.0]	80.5 [60.0–181.5]	70.0 [37.0–181.8]	NS
Male gender (%)	12 (80)	4 (50)	20 (50)	NS
Gestational age (wk)	37.0 [33.0–39.0]	39.4 [38.3–40.0]	39.0 [37.0–41.0]	<0.05*
Prematurity (%)	7 (47)	1 (13)	8 (20)	NS
Birth weight (kg)	2.6 [2.1–3.1]	3.2 [2.8–4.1]	3.2 [2.8–4.0]	<0.05†
Breast-feeding (%)	7 (47)	3 (37.5)	25 (62.5)	NS
Symptomatic (d)	0 (0)	4.0 [3.0–5.0]	4.0 [3.0–5.0]	NS
Duration O2 (d)	0 (0)	0 (0)	4.5 [2.0–8.8]	<0.001‡
Duration MV (d)	0 (0)	0 (0)	0 [0–8.8]	<0.05§
Corticosteroids (%)	0 (0)	0 (0)	0 (0)	NS
Antibiotics (%)	0 (0)	4 (50)	22 (55)	NS
Palivizumab (%)	0 (0)	0 (0)	0 (0)	NS
Hospitalization (d)	0 (0)	4.0 [2.0–7.5]	8.0 [5.0–11.0]	<0.001

*Healthy vs. no oxygen and oxygen $P < 0.05$. †Healthy vs. oxygen $P < 0.01$. ‡Oxygen vs. no oxygen $P < 0.001$. §Oxygen vs. no oxygen $P < 0.001$. ¶Healthy vs. no oxygen and oxygen $P < 0.001$, oxygen vs. no oxygen $P < 0.05$.

CD14 Expression on Monocytes Is Increased During RSV Infection

We next investigated whether monocytes during RSV infection are phenotypically different. The expression level of the surface marker CD14 was measured, and we observed an increased expression of CD14 on all 3 subpopulations of monocytes during the acute phase of infection (**Figure 7.3A**). This increased expression of CD14 was present in all RSV-infected infants, and there was no difference in the expression of CD14 between the disease severity groups (**Figure 7.3B**). We observed no correlation between CD14 expression and age (data not shown).

HLA-ABC Expression on Monocytes Is Increased During RSV Infection

Antigen-presenting molecules on monocytes have been implicated in disease occurrence and progression. To determine whether monocytes contained increased amounts of antigen-presenting molecules during RSV infection, we first measured the expression level of HLA-ABC on all subsets of monocytes. We observed an increased expression of HLA-ABC on all 3 subpopulations of monocytes during RSV infection (**Figure 7.4A**). There was no difference in the expression of HLA-ABC between disease severity groups after RSV infection (**Figure 7.4B**). We observed no correlation between HLA-ABC expression and age (data not shown).

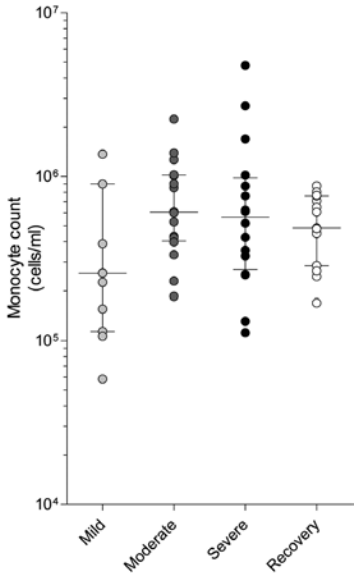

Figure 7.1 Monocyte count in the peripheral blood determined by blood smear analysis. Monocyte counts during the acute phase of RSV infection and during the recovery phase. Values are depicted as medians ± interquartile range. Testing was performed with Kruskal-Wallis and if significant followed by the Mann-Whitney *U* test.

Low HLA-DR Expression on Monocytes Is Correlated With Increased Disease Severity and Prolonged Hospitalization

Low HLA-DR expression on monocytes has been associated with increased disease severity of bacterial infections. We determined the level of HLA-DR expression on monocytes to correlate HLA-DR expression with disease severity in RSV infection. The HLA-DR expression on classical and intermediate monocytes was significantly lower during RSV infection (**Figure 7.5A**). The expression of HLA-DR on classical and intermediate monocytes correlates with diseases severity. Monocytes from RSV-infected infants with- out oxygen treatment have comparable expression levels of HLA- DR compared with healthy controls (**Figure 7.5B**). Severe RSV-infected infants requiring oxygen therapy have lower expression levels of HLA-DR compared with both RSV-infected infants without oxygen therapy and healthy controls (**Figure 7.5B**).

We observed no correlation between HLA-DR expression and age, suggesting that age is most likely not a potential confounder for the reduced HLA-DR expression in severe RSV infections (data not shown). As a second parameter, we determined whether HLA-DR expression was correlated with the duration of hospitalization. Reduced HLA-DR expression on classical monocytes significantly correlated with prolonged hospitalization (**Figure 7.5C**). These data indicate that low HLA-DR expression is

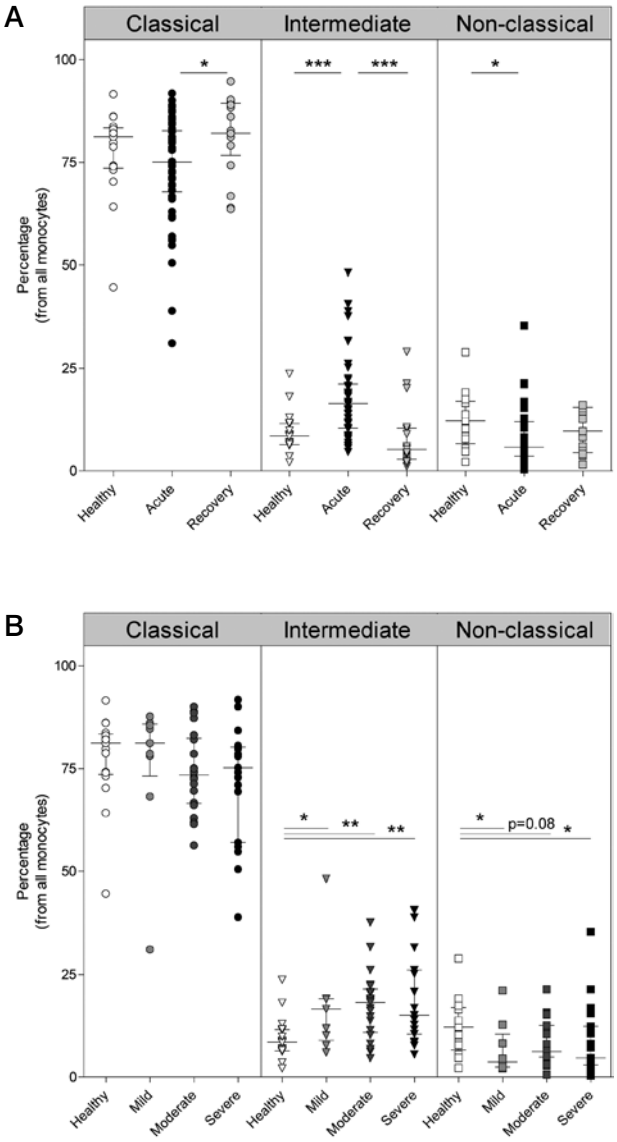

Figure 7.2 The percentage of intermediate monocytes is increased during RSV infection. **A**, Percentages of monocyte populations in the acute phase of RSV infection compared with healthy controls and the recovery phase. **B**, Percentages of monocyte populations in infants categorized by disease severity. Values are depicted as medians ± interquartile range. Testing was performed with Kruskal-Wallis and if significant followed by the Mann-Whitney *U* test (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).

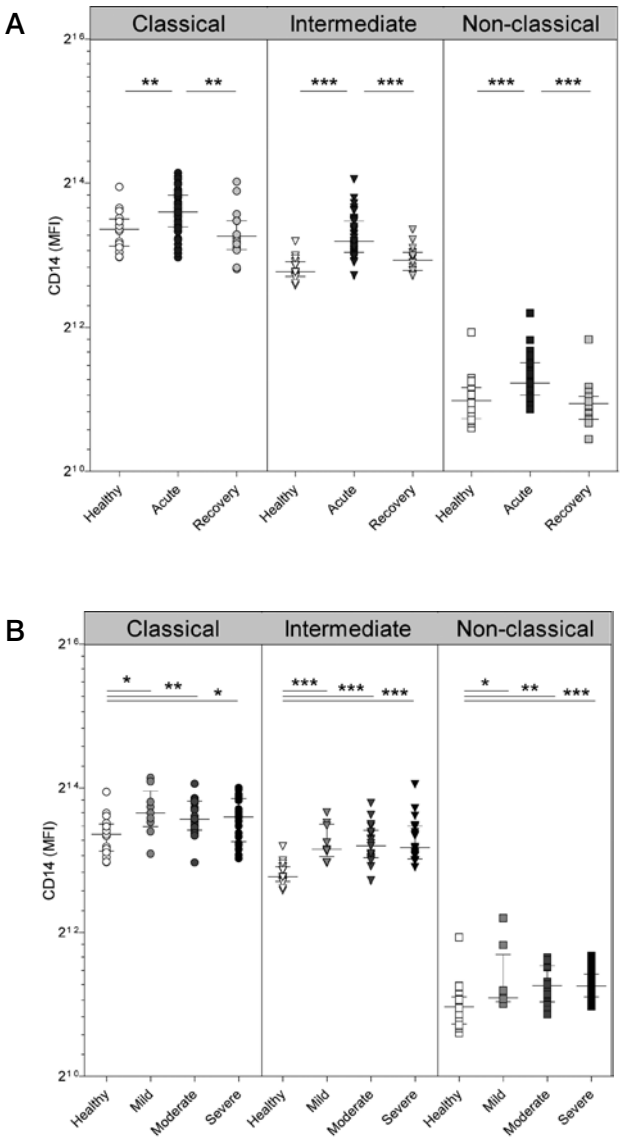

Figure 7.3 CD14 expression on circulating monocytes is increased during RSV infection. **A**, CD14 expression on monocytes during the acute phase of RSV infection compared with healthy controls and the recovery phase. **B**, CD14 expression on monocyte populations in infants categorized by disease severity. Values are depicted as medians ± interquartile range. Testing was performed with Kruskal-Wallis and if significant followed by the Mann-Whitney *U* test (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).

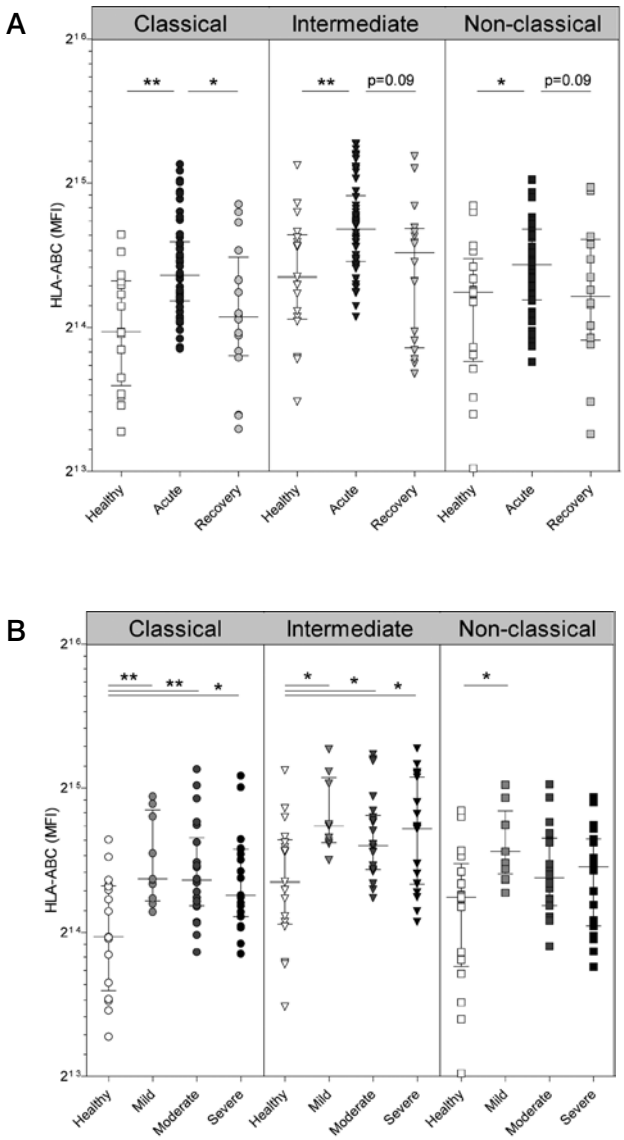

Figure 7.4 HLA-ABC expression on circulating monocytes is increased during RSV infection. **A**, HLA-ABC expression on monocytes during the acute phase of RSV infection compared with healthy controls and the recovery phase. **B**, HLA-ABC expression on monocyte populations in infants categorized by disease severity. Values are depicted as medians ± interquartile range. Testing was performed with Kruskal-Wallis and if signify followed by the Mann-Whitney *U* test (*$P < 0.05$, **$P < 0.01$).

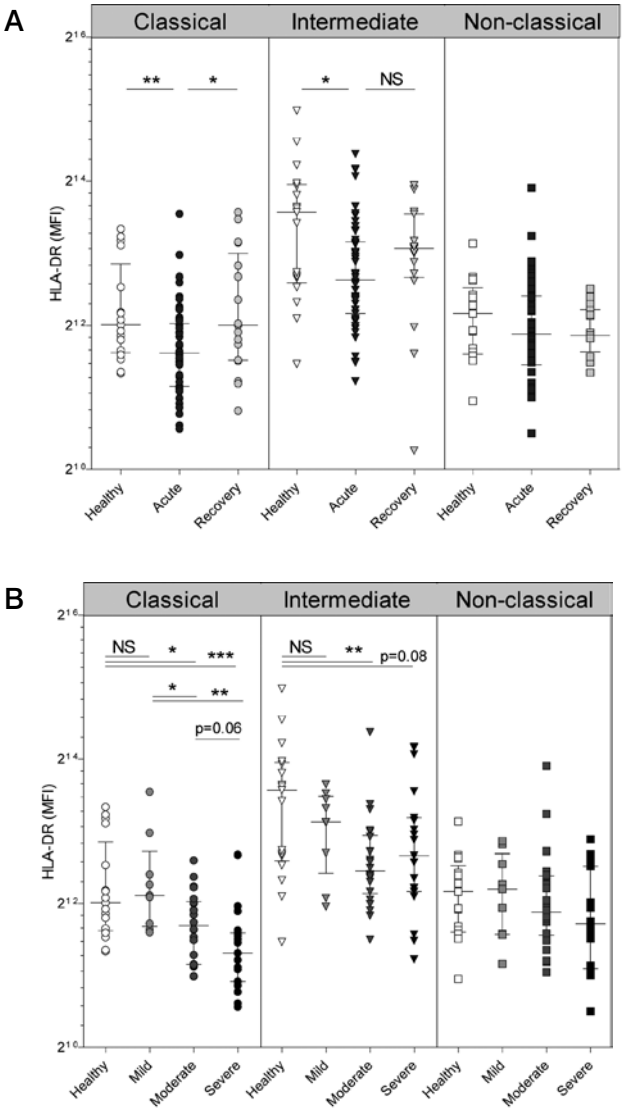

Figure 7.5 Low HLA-DR expression is correlated with increased disease severity and prolonged hospitalization. **A**, HLA-DR expression on monocytes during the acute phase of RSV infection compared with healthy controls and the recovery phase. **B**, HLA-DR expression on monocyte populations in infants categorized by disease severity. Values are depicted as medians ± interquartile range. **C**, Correlation between duration of hospitalization and HLA-DR expression on classical monocytes. Testing was performed with Kruskal-Wallis and if significant followed by Mann-Whitney *U* test. Testing for correlation was performed with the Pearson test (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).

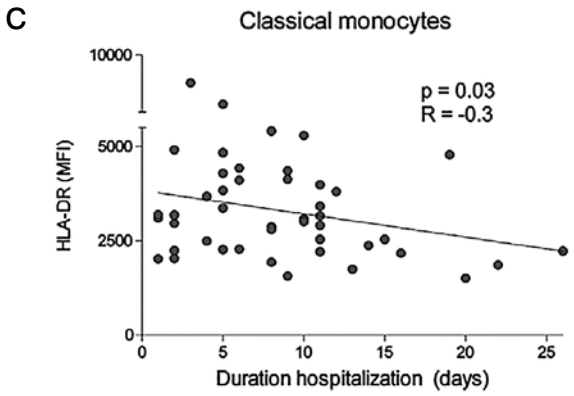

Figure 7.5 Continued.

associated not only with the presence of RSV infection but also with increased disease severity, oxygen treatment and prolonged hospitalization.

Severe RSV Infections Are Associated With Impaired In Vitro IL-10 Production Upon Stimulation With LPS

Besides low HLA-DR expression, impaired proinflammatory cytokine production has been associated with disease severity in bacterial infections. Cytokine production of PBMCs from healthy and RSV-infected infants upon in vitro stimulation with LPS was determined to investigate whether decreased LPS responsiveness was associated with severe RSV infections. No significant difference in TNF production upon LPS stimulation was observed between the severity groups and the healthy controls (**Figure 7.6A**). PBMCs from RSV-infected infants without oxygen therapy produced similar amounts of IL-10 compared with healthy controls (**Figure 7.6B**). However, PBMCs from RSV-infected infants that require oxygen therapy produced significantly lower amounts of IL-10 upon LPS stimulation compared with healthy controls (**Figure 7.6B**). There was no correlation between age and either TNF or IL-10 production (data not shown).

Discussion

This study shows an increased percentage of circulating intermediate monocytes during RSV infection. An increased expression of CD14 and HLA-ABC was present during RSV infections. Conversely, reduced HLA-DR expression on monocytes was present during RSV infection and low HLA-DR expression was correlated with increased disease severity and prolonged hospitalization. Finally, an impaired in vitro IL-10 production of PBMCs from infants with severe RSV infections compared with healthy controls was observed. Analysis of our data indicates that the immune profile of monocytes changes

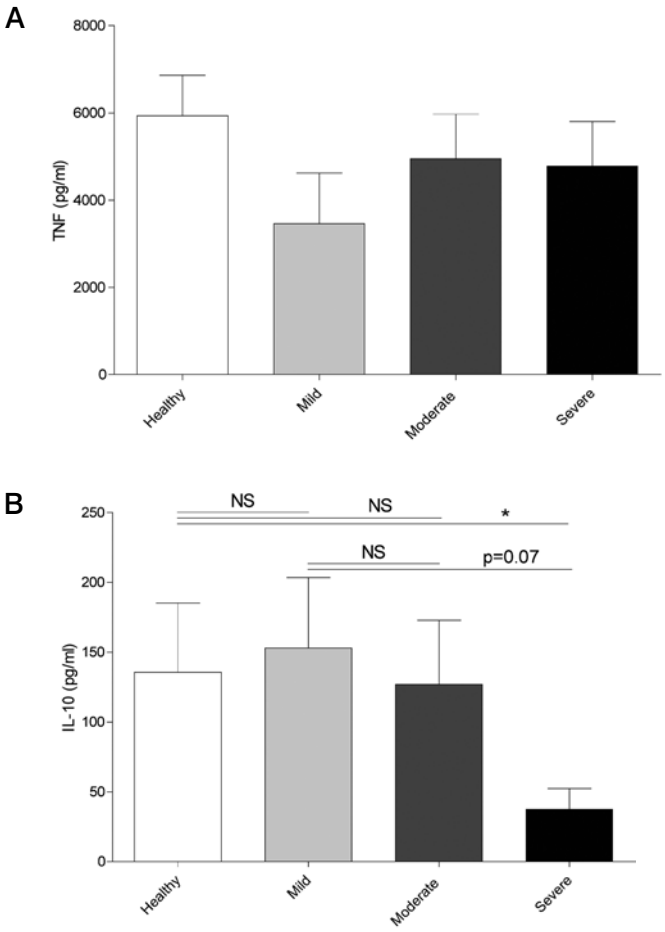

Figure 7.6 Severe RSV infections are associated with impaired in vitro IL-10 production upon stimulation with LPS. Cytokine production of PBMCs from healthy controls and RSV-infected infants, categorized by disease severity, after 20-hour stimulation with LPS for (**A**) TNF and (**B**) IL-10. Values are represented as means with SEM. Testing was performed with the Mann-Whitney *U* test (*$P < 0.05$).

during RSV infection and that, in particular, low expression of HLA-DR correlates with the increased disease severity of RSV infection.

The observed increase of intermediate monocytes is in line with studies investigating pediatric bacterial and viral infections.[20,21] This is the first report implicating an increase of intermediate monocytes as a consequence of RSV infection. These data suggest that the increase of intermediate monocytes is applicable to many infectious diseases,

including viral respiratory tract infections. $CD14^{++}CD16^{+}$ monocytes are termed proinflammatory because of their ability to produce high amounts of cytokines. Next, they possess high levels of HLA-DR and are potent inducers of T cell proliferation.[22,23] Previous data suggest that these activated monocytes migrate to inflamed tissues to locally support the ongoing T cell activation.[24] In the context of RSV, the increased percentage of intermediate monocytes might be a reflection of immune activation and these monocytes may be subsequently attracted to the inflamed lung tissue to exert local antiviral immunity.

Besides the increase of intermediate monocytes, all subsets of monocytes during RSV infection in our cohort expressed higher levels of CD14. Maus *et al* showed that monocytes are recruited from the blood stream to become alveolar monocytes during infection.[25,26] These recruited monocytes have a high expression of CD14, exert enhanced cytokine production and are able to replace resident alveolar macrophages. Next, viral proteins are able to promote a Th1 response via CD14.[27,28] Polarization toward aTh2-dominated response is present during RSV infections, and therefore, the increased expression of CD14 on monocytes might be a defense mechanism against RSV.[16] Whether the high CD14-expressing monocytes in our cohort in the peripheral blood during RSV infection are indeed recruited to the inflamed lung tissue and polarize the local immune response against RSV cannot be determined in this study.

In our cohort, expression levels of HLA-ABC on monocytes are higher during infection, whereas expression levels of HLA-DR on monocytes are suppressed. HLA-ABC and HLA-DR expression on monocytes functions to bind peptide fragments from pathogens and present them for recognition by, respectively, $CD8^{+}$ and $CD4^{+}$ T cells. Increased expression of HLA-ABC suggests that during RSV infection, circulating monocytes are primed for antigen presentation. However, HLA-DR expression on monocytes is reduced, and this is most pronounced in patients with severe RSV infections. Low HLA-DR expression has been associated with immunoparalysis and disease severity in bacterial infections.[11,29,30] Our data suggest that RSV exerts a more pronounced inhibitory effect on HLA-DR expression during severe RSV infections. Reduction of HLA-DR expression by RSV on monocytic cells has already been observed in vitro.[31] Connolly *et al* describe a direct effect of RSV infection on hampering of HLA-DR surface translocation.[32] However, direct blocking of HLA- DR surface translocation as the underlying mechanism of reduced HLA-DR expression in vivo is unlikely as viremia has only sporadically been detected during RSV infection. Soluble inflammatory mediators present in the blood could be responsible for the observed reduced HLA-DR expression in infants with severe RSV infections. For instance, high cortisol levels have been associated both with severe RSV infections and reduced HLA-DR expression and might explain the reduced HLA-DR expression in severe RSV infections.[33,34] The correlation between reduced HLA-DR expression and prolonged hospitalization confirms our conclusion regarding HLA-DR and disease severity. The decision whether to admit RSV-infected infants or send them home is mainly based on the need for oxygen treatment. For many patients presenting at the emergency room, it is difficult

to determine the course of disease and thereby the future need for oxygen treatment. Therefore, the difference in HLA-DR expression between infants without oxygen treatment and infants with oxygen treatment may have important clinical implications and could help the clinician to decide whether to admit a patient with RSV bronchiolitis. Transcriptional profiling of blood from RSV-infected infants showed a suppression of important T cell genes, in particular, in younger infants, and $CD4^+$ T cells are lower in severe RSV infections compared with nonsevere RSV infections.[19,35] Our observed immune profile with reduced HLA-DR expression on monocytes during severe RSV infections might explain the low $CD4^+$ T cell responses seen in severe RSV infections.[19] Further research dissecting the mechanism of reduced HLA-DR expression will give more insight in the immunomodulatory effects of RSV.

A second hallmark of immunoparalysis is a reduced TNF production upon stimulation with LPS, which was not observed in our cohort of infants with RSV infections. We conclude that although HLA-DR expression is decreased during RSV infection, there is no apparent immunoparalysis as seen in bacterial infections. Mella *et al* observed a correlation between impaired LPS responsiveness and severe RSV infections by stimulation of whole blood.[36] Although the stimulation of whole blood might include more cells involved in clinical disease, stimulation of PBMCs has proven to be useful to decipher the mechanisms between cytokine release and disease severity.[37]

We observed a significantly impaired IL-10 production upon LPS stimulation by PBMCs from infants with severe RSV infections compared with healthy controls. The role of IL-10 is contrary as it has been associated with either the development of asthma or the protective immunity in a murine model.[38–40] IL-10 has been implicated as an important cytokine that influences immunopathology in RSV infection by limiting T-cell-mediated lung injury.[41] Our observed impairment of anti-inflammatory cytokine production by PBMCs indicates a shift toward a less anti-inflammatory and a more proinflammatory profile in vivo.

In this study, we were able to include an age- and sex- matched control group with infants who required an inguinal hernia operation. Although age and sex were matched between the control group and the RSV-infected infants, the gestational age in the control group was significantly lower compared with the RSV- infected infants. We believe that this difference of 2 days would not significantly impact the matching between healthy controls and RSV-infected infants. Low gestational age, in particular $<$32 weeks of age, is a risk factor for severe RSV infection. When comparing prematurity, defined as gestational age $<$37 weeks, there are no significant differences between the groups. Therefore, the difference in gestational age is unlikely to interfere with the results observed in severe RSV-infected infants.

In summary, striking differences are found in monocytes of infants with RSV infections. The increase of intermediate monocytes, the high CD14 expression and the high HLA-ABC expression suggest that activation of monocytes occurs during RSV infection, which might result in recruitment of monocytes to the local site of infection. The reduced expression of HLA-DR combined with reduced in vitro responsiveness

during RSV infection, however, might be an important indicator of immunosuppression of monocytes during severe RSV infections.

Acknowledgments

The authors thank all parents, children, Mariette Las and the staff of the 2 pediatric wards for their contributions to this study. Mediq Tefa provided the Cheiron Dynamic II apparatus on loan, to enable us to perform nasal washes during home visits. Furthermore, they thank E. Voorbrood and P. Ruijs from the Laboratory of Hematology at the Radboud university medical center for analysis of the blood smears.

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8

Use of MMP-8 and MMP-9 to assess disease severity in children with viral lower respiratory tract infections

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Abstract

Matrix metalloproteinases (MMPs) play an important role in respiratory inflammatory diseases, such as asthma and chronic obstructive pulmonary disease. It was hypothesized that MMP-8 and MMP-9 may function as biological markers to assess disease severity in viral lower respiratory tract infections in children. MMP-8 and MMP-9 mRNA expression levels in peripheral blood mononuclear cells (PBMCs) and granulocytes obtained in both the acute and recovery phase from 153 children with mild, moderate, and severe viral lower respiratory tract infections were determined using real-time PCR. In addition, MMP-8 and MMP-9 concentrations in blood and nasopharyngeal specimens were determined during acute mild, moderate, and severe infection, and after recovery using ELISA. Furthermore, PBMCs and neutrophils obtained from healthy volunteers were stimulated with RSV, LPS (TLR4 agonist), and Pam3Cys (TLR2 agonist) in vitro. Disease severity of viral lower respiratory tract infections in children is associated with increased expression levels of the MMP-8 and MMP-9 genes in both PBMCs and granulocytes. On the contrary, in vitro experiments showed that MMP-8 and MMP-9 mRNA and protein expression in PBMCs and granulocytes is not induced by stimulation with RSV, the most frequent detected virus in young children with viral lower respiratory tract infections. These data indicate that expression levels of the MMP-8 and MMP-9 genes in both PBMCs and neutrophils are associated with viral lower respiratory tract infections disease severity. These observations justify future validation in independent prospective study cohorts of the usefulness of MMP-8 and MMP-9 as potential markers for disease severity in viral respiratory infections.

Introduction

Respiratory viral infections are an important cause of hospitalization among children younger than 5 years of age with estimated population-based hospitalization rates of 1–2%.[1-3] Human respiratory syncytial virus (RSV) is the most commonly identified virus with detection rates up to 40–85% in infants hospitalized for respiratory infections during winter epidemics,[4-6] The clinical manifestations range from a simple common cold to severe lower respiratory tract symptoms requiring mechanical ventilation. About 6– 11% of the children admitted to hospital with RSV infection require intensive care admission.[7,8] Up to 35% of the children hospitalized with bronchiolitis did not receive any supportive intervention.[9] On the other hand, it is crucial to avoid discharge of those children who may experience clinical deterioration. Among children sent home with the diagnosis bronchiolitis, 4.6–6.8% required hospitalization later on during infection.[10,11] Biomarkers to assess severity of viral lower respiratory tract infections, in particular RSV infection, may be helpful to clinicians in the decision whether a child needs to be hospitalized.

Lung injury during severe RSV infection is thought to be mediated by both direct cytotoxic effects of the virus and the result of the induced inflammation. Pathologic features of severe RSV infection include extensive bronchiolar epithelial destruction, peribronchial lymphocyte infiltration, necrosis of bronchial epithelium, and mucus plugs in the small bronchioles.[12,13] Matrix metalloproteinases (MMPs) are family of zinc endo-peptidases capable of degrading components of the cellular matrix, and consequently, are suggested to be important in several diseases associated with tissue remodeling. Pronounced increase in their expression is thought to be associated with a variety of inflammatory disease, including respiratory diseases.[14]

MMPs play a role in cellular migration of neutrophils, lymphocytes, and other immune cells to the lungs by degrading extracellular matrix, but also have pro- and anti-inflammatory properties. The activity of MMPs is regulated through binding to tissue inhibitor of metalloproteinases (TIMPs) leading to in- activation. An imbalance in production and activation, or inactivation by TIMPs might augment airway inflammation through direct or indirect effects upon signalling pathways that influence migration of leukocytes through the tissues.[14,15] Increased concentrations and activity of MMP-8 and MMP-9 have been observed in respiratory samples obtained from adults and children with acute lung injury and pneumonia[16-19] as well as in chronic lung diseases such as asthma.[20,21] In addition, a relation between MMP-9 concentrations and disease severity of pneumonia [16,18] and asthma [22,23] has been described.

Yeo et al. have reported that MMP-9 protein expression is increased in human airway epithelial cell lines infected with RSV.[24] In addition, MMP-9 gene expression is increased in the lungs of RSV-infected mice.[25] Another study demonstrated that nasopharyngeal samples from infants infected with RSV and parainfluenza virus (PIV) contain increased MMP-9 and TIMP-1 concentrations.[26]

In the current study, it was hypothesized that MMP-8 and MMP-9 gene expression levels, and consequently, MMP-8 and MMP-9 plasma concentrations may function as biomarkers for disease severity in viral lower respiratory tract infections.

Methods

Study Design

Children younger than 5 years of age with laboratory confirmed viral lower respiratory tract infections were prospectively included during three consecutive winter seasons (November–April in the years 2006–2009). Patients with congenital heart or lung disease, known immunodeficiency's or glucocorticoid use were excluded. Viral lower respiratory tract infections was defined as an acute infection of the lower airways, characterized by increased respiratory effort (tachypnea and/or use of accessory respiratory muscles and/or expiratory wheezing and/or crackles and/or apnea) in combination with a confirmed viral etiology by multiplex real-time polymerase chain reaction (RT-PCR) on nasopharyngeal washes as described previously.[27] The multiplex RT-PCR assay detect 15 different viral pathogens; influenza virus types A and B, coronavirus 229E and OC43, human bocavirus, enterovirus, adenovirus, parechovirus, PIV types 1–4, human metapneumovirus, rhinovirus (RV), and RSV.

Written informed consent was obtained from all parents and the study was approved by the Committee on Research involving Human Subjects of the University Nijmegen Medical Centre. Within 24 hr after admission a blood sample and nasopharyngeal aspirate was collected and parents from hospitalized children were asked permission to draw a second blood sample and nasopharyngeal aspirate 4–6 weeks after admission. Medical history, demographics, and clinical parameters were collected from questionnaires and medical records. Patients were classified into three different groups based on severity of disease. Children without hypoxia or severe feeding problems were allocated in the mild group, those requiring hospitalization for supplemental oxygen (oxygen saturations $<$93%) and/or nasogastric feeding in the moderate group and children requiring mechanical ventilation in the severe group.

Sample Collection

A nasopharyngeal aspirate was collected by introducing a catheter, connected to a collection tube and an aspiration system, into the nasopharyngeal cavity. Then, 1.5 ml of saline was instilled into the catheter and, while slowly retracting the catheter, the nasopharyngeal fluid was aspirated in a collection tube. Afterwards the catheter was flushed with 1 ml of saline and added to the collection fluid. The samples were kept cold and immediately transferred to the laboratory. The nasopharyngeal aspirate was centrifuged at 500*g* for 10 min at 48C to spin down the mucus and cells, after which the supernatant was frozen at -80C.

Five millilitres of blood was collected into sodium heparin tubes and directly transferred to the laboratory. A thin blood smear was prepared and stained with (May-Grunwald-) Giemsa to determine the percentages of granulocytes and PBMCs. PBMCs were obtained by density gradient centrifugation (Lymphoprep[1]; Axis Shield, Oslo, Norway) and stored in Trizol at -80C for RNA isolation. Plasma samples were stored at -80C for ELISAs

Quantitative mRNA Expression of MMP-8 and MMP-9 in PBMCs and Granulocytes

RNA from PBMC and granulocytes was extracted using Trizol (Invitrogen Life Technologies, Bleiswijk, The Netherlands) according to the manufacturers' protocol. Subsequently, a clean-up was performed on total RNA with the RNeasy Minikit (Qiagen, Venlo, The Netherlands) according to the manufacturers' instructions. Total RNA (2 mg, measured with spectrophotometry, Nanodrop, Wilmington) was reverse transcribed using a high-capacity cDNA reverse transcription kit according to the manufacturers' instructions (Applied Biosystems, Foster City, CA) and cDNA was stored at -20C. The relative gene expression was measured with SYBR Green PCR Mastermix (Applied Biosystems; P/N 4367659) on the ABI 7500 Fast Real Time PCR system using standard program and software. After 40 repetitions a dissociation curve was performed as control for the specificity of the PCR reaction. The following primers were used: hActin F: CGTCACACTTCATGATGGAGTTG, hActin R: CTTCCTTCCTGGGCATGGA; hMMP-9 F: GCCCCCCTTGCATAAGGA, hMMP-9 R: CAGGGCGAGGACCATAGAG; and hMMP-8 F: CCAGTTTGACATTTGATGCTATCAC, hMMP-8 R: CTGAGGATGCCTTCTCCAGAA. All reactions were performed in duplo. Actin was used as reference gene. After a quality check (melting temp, curve of reaction, and standard deviation Ct) the DCt of the MMP-8 and MMP-9 to actin was calculated and expressed as relative expression.

MMP-8, MMP-9, and TIMP-1 Concentrations in Plasma and Nasopharyngeal Washes

Concentrations of total MMP-8 and MMP-9 in plasma, nasopharyngeal aspirate, and supernatants of cell stimulation assay were measured by ELISA according to the manufacturers' protocol (DuoSet, R&D systems, Abingdon, UK). In addition, TIMP-1 concentrations in plasma were determined as described above.

In Vitro Stimulation of PBMCs and Neutrophils From Healthy Volunteers

After informed consent, blood was drawn from healthy volunteers and collected in EDTA tubes. Blood was diluted 1:1 with pyrogene-free PBS (Lonza, Basel, Switzerland). PBMCs and granulocytes were obtained by density gradient centrifugation (Lymphoprep[1]; Axis Shield). After washing, PBMCs were brought at a concentration of 5 x 10^6 cells/ml in serum-free RPMI (Gibco, Invitrogen, Paisley, UK) with 100 U/ml of penicilin/streptavidin (Gibco, Invitrogen). Granulocytes were purified by lysing the red blood cells (0.155 M

NH4Cl, 0.0001 M Na2EDTA and 0.01 M KHCO3), and, after washing, granulocytes were suspended at a concentration of 5 x 10^6 cells/ml in RPMI supplemented with 0.5% human serum albumin (Sanquin, Amsterdam, The Netherlands). Mononuclear cells (5 x 10^5 in 100 ul) were added to round-bottom 96-well plates and stimulated with either 100 ul culture medium (negative control), 1 ng/ ml LPS (*Escherichia coli* serotype 055:B5, Sigma– Aldrich, purified as described previously [28] or MOI 1 of RSV A2 (kindly provided by Dr. R. de Swart, Erasmus MC, Rotterdam, The Netherlands). RSV A2 was cultured in HeLa cells and purified by ultracentrifuge over a sucrose 30% gradient. After incubation for 24 hr at 378C and 5% CO2. supernatant was collected and stored at -80C.
Neutrophils (5 x 10^5 in 100 ul) were stimulated and incubated in the same way for 4 hr and supernatant was stored at -80C. Apoptosis was determined on the FACScalibur by Annexin V apoptosis detection kit (BD) according to the manufacturers' instructions and no differences between stimuli were found after 4 hr.

Statistics

Values are expressed as percentages for categorical variables and as mean and standard error (SE) or median and interquartile range (IQR) for continuous variables. For variables that were not normally distributed, Kruskal–Wallis test was performed to compare continuous variables followed by Mann–Whitney *U*-tests for individual comparisons. Chi-squared tests were performed to compare categorical data. A two-sided value of $P < 0.05$ was considered statistically significant.

Results

Patient Characteristics

In total, 153 patients were included. In 109 patients (71%) RSV was detected. RSV positive children were significantly younger than RSV negative children. No other significant differences were observed between these groups (**Table 8.1**).
A total of 54, 60, and 39 children were classified as having mild, moderate, and severe disease, respectively. Patients with severe disease were significantly younger compared to those with mild disease (105 days vs. 278 days; $P < 0.05$). More prematurely born children were observed in the severe group compared to the mild and moderate group. No other significant differences in clinical parameters were found between the different severity groups (**Table 8.2**). In addition, total leukocytes and neutrophil counts were comparable between all groups.

Disease Severity Is Associated With Increased Gene Expression Levels of MMP-8 and MMP-9 in Both Granulocytes and PBMCs

During acute viral infection we observed increased expression of the MMP-8 and MMP-9 genes in both

Table 8.1 Patient Characteristics.

	Total (n= 153)	RSV+ (n= 109)	RSV– (n= 44)	*P*-value
Age (days ± SE)	206 ± 26	149 ± 20	347 ± 72	<0.001
Male	95 (62%)	70 (64%)	25 (57%)	NS
Prematurity	21 (14%)	16 (15%)	5 (11%)	NS
Family history of atopy	82 (57%)	56 (54%)	26 (63%)	NS
Symptomatic days before presentation (days ± SE)	5.5 ± 0.4	5.2 ± 0.4	6.3 ± 1.1	NS

Data are presented as number (%), unless otherwise specified. For variables that were not normally distributed, Kruskal–Wallis test was performed to compare continuous variables followed by Mann–Whitney *U*-tests for individual comparisons. Chi-squared tests were performed to compare categorical data. A two-sided value of $P < 0.05$ was considered statistically significant. NS, not significant, SE, standard error.

PBMCs and granulocytes compared to recovery. No differences in gene expression of MMP-8 and MMP-9 in both PBMCs and granulocytes were found between RSV positive and RSV negative children during acute infection. In general, gene expression of the MMP-9 gene was higher in granulocytes than in PBMCs. For MMP-8, the same trend was noticed (**Figure 8.1A,B and 2A,B**).

Increased disease severity was associated with higher expression levels of the MMP-8 and MMP-9 genes in both PBMCs and granulocytes. To determine whether this association was dependent on the type of virus, RSV positive and RSV negative children were analyzed separately. For RSV positive patients, the same association was found between disease severity and gene expression levels (**Figure 8.1C,D and 2C,D**). For RSV negative patients, MMP-8 and MMP-9 gene expression was higher in children with severe disease compared to those with mild. In addition, RSV negative children with severe disease had higher expression levels of the MMP-8 gene in both PBMCs and granulocytes compared to those with moderate disease (data not shown).

Disease Severity Is Associated With Increased MMP-8 Plasma Levels

The plasma concentration of MMP-8 was increased during acute RSV infection compared to recovery. In RSV negative patients this difference was not significant (**Figure 8.3A**). Higher MMP-8 plasma concentrations were found in children with severe and moderate disease compared to those with mild disease (**Figure 8.4A**). In nasopharyngeal washes, the concentration of MMP-8 was increased during acute RSV infection compared to recovery washes (**Figure 8.3D**). No significant differences of MMP-8 concentrations in the nasopharyngeal washes were observed between the different severity groups (**Figure 8.4D**). In both RSV positive as RSV negative patients MMP-9 concentrations in the nasopharyngeal washes were increased during infection (**Figure 8.3E**). Children with moderate disease had increased MMP-9 concentrations in nasopharyngeal washes compared to those with mild disease. However, no significant

differences in MMP-9 concentrations were observed in children with severe disease compared to those with mild and moderate disease (**Figure 8. 4E**). TIMP-1 concentrations in plasma or nasopharyngeal washes were not increased during acute infection and there was no correlation with disease severity (**Figure 8.3C and 4C**). The ratio between MMP-9 and TIMP-1 plasma concentrations, an indicator for enzyme activity, was not increased during acute RSV infection. This ratio was significant higher in the recovery plasma of RSV negative patients compared to the acute samples (**Figure 8.3F**). No relation between disease severity and the plasma MMP-9 and TIMP-1 ratio was found (**Figure 8. 4F**).
No differences in plasma or nasopharyngeal levels of MMP-8, MMP-9, and TIMP-1 plasma concentrations were observed between RSV positive and RSV negative children during acute viral respiratory infection. In general, MMP-8 and MMP-9 concentrations were higher in nasopharyngeal samples compared to plasma.

Table 8.2 Patient Characteristics for Mild, Moderate, and Severe Infections

	Total (n = 153)	Mild (n = 54)	Moderate (n = 60)	Severe (n = 9)	*P*-value
Age (days)	206 ± 26	278 ± 52	206 ± 38	105 ± 36	<0.01
Male (%)	95 (62%)	32 (59%)	36 (60%)	27 (69%)	NS
Prematurity (%)	21 (14%)	5 (9%)	6 (10%)	10 (26%)	<0.05
Family history of atopy (%)	82 (57%)	27 (52%)	36 (62%)	19 (55%)	NS
Symptomatic days before presentation	5.5 ± 0.4	7.0 ± 1.1	4.8 ± 0.3	4.6 ± 0.4	NS
RSV (%)	109 (71%)	35 (65%)	45 (75%)	29 (74%)	NS
Leukocytes counts	8.7 ± 0.7	9.8 ± 1.1	8.6 ± 1.0	8.2 ± 1.4	NS
Neutrophil counts	3.5 ± 0.5	3.1 ± 0.7	2.9 ± 0.7	4.3 ± 1.1	NS

Data are presented as percentages or mean ± standard error (SE). Cell counts are given as *10E6 cells/ml ± SE. For variables that were not normally distributed, Kruskal–Wallis test was performed to compare continuous variables followed by Mann–Whitney *U*-tests for individual comparisons. Chi-squared tests were performed to compare categorical data. A two-sided value of $P < 0.05$ was considered statistically significant. NS, not significant.

MMP-9 Plasma Concentrations Are Correlated With the Number of Granulocytes

MMP-9 plasma concentrations correlated with the number of granulocytes measured during acute RSV infection (Pearson's correlation coefficient 0.33; P ¼ 0.019). No correlation was found between the number of granulocytes and MMP-8 plasma and nasopharyngeal concentrations and MMP-9 nasopharyngeal concentrations (data not shown). Furthermore, there was no correlation between symptomatic days before presentation and levels of MMP-8 and MMP-9 gene expression or concentration of the protein in plasma and nasopharyngeal washes (data not shown).

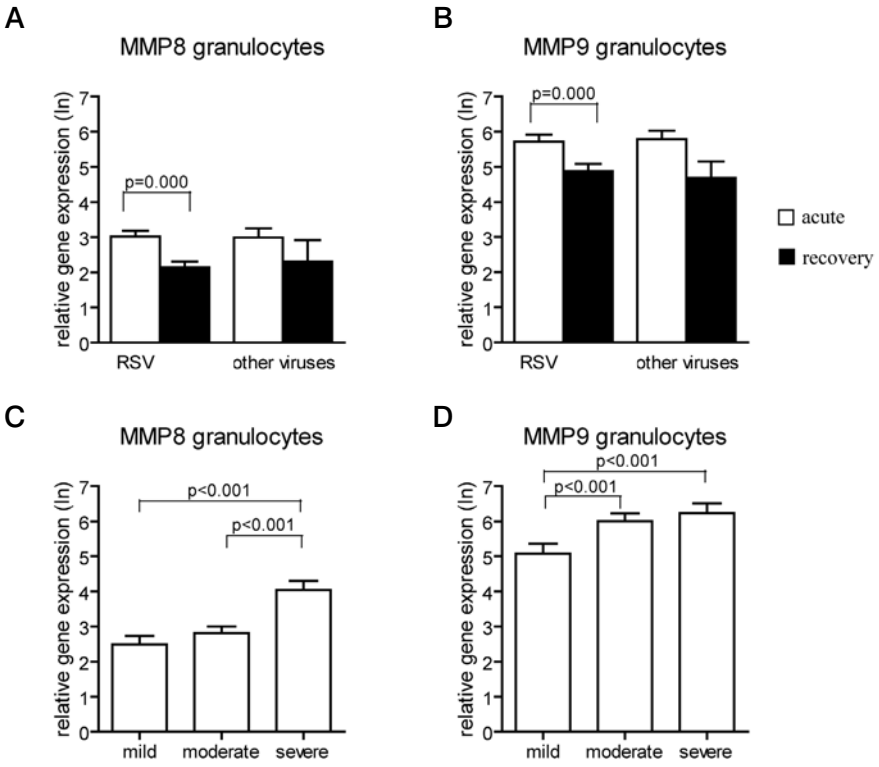

Figure 8.1 Gene expression levels of MMP-8 and MMP-9 in granulocytes from children with viral lower respiratory tract infections. Relative gene expression levels (mean ± standard error) of MMP-8 (**A**) and MMP-9 (**B**) in PBMCs from children during acute RSV positive and RSV negative viral lower respiratory tract infections and after recovery of infection. Relative gene expression levels of MMP-8 (**C**) and MMP-9 (**D**) for RSV positive children with mild, moderate, and severe disease. Mann–Whitney *U*-test was performed to compare children infected by RSV and other viruses. Paired analyses (Wilcoxon) were performed to compare acute and recovery samples. A two-sided value of $P < 0.05$ was considered statistically significant.

MMP-8 and MMP-9 mRNA and Protein Expression by PBMCs and Neutrophils Is Not Induced by RSV In Vitro

To investigate whether the source of plasma MMP- 8 and MMP-9 during RSV infection was the result of direct interaction of PBMCs or neutrophils with RSV, PBMCs and neutrophils were stimulated with RSV in vitro. Stimulation of PBMCs with LPS (TLR4 agonist) induced MMP-9 secretion, whereas stimulation with RSV had no effect. None of the stimuli induced MMP- 8 secretion by PBMC (Fig. 5A). Stimulation of PBMCs with RSV did not result in increased gene expression of MMP-8 and only a moderate increase

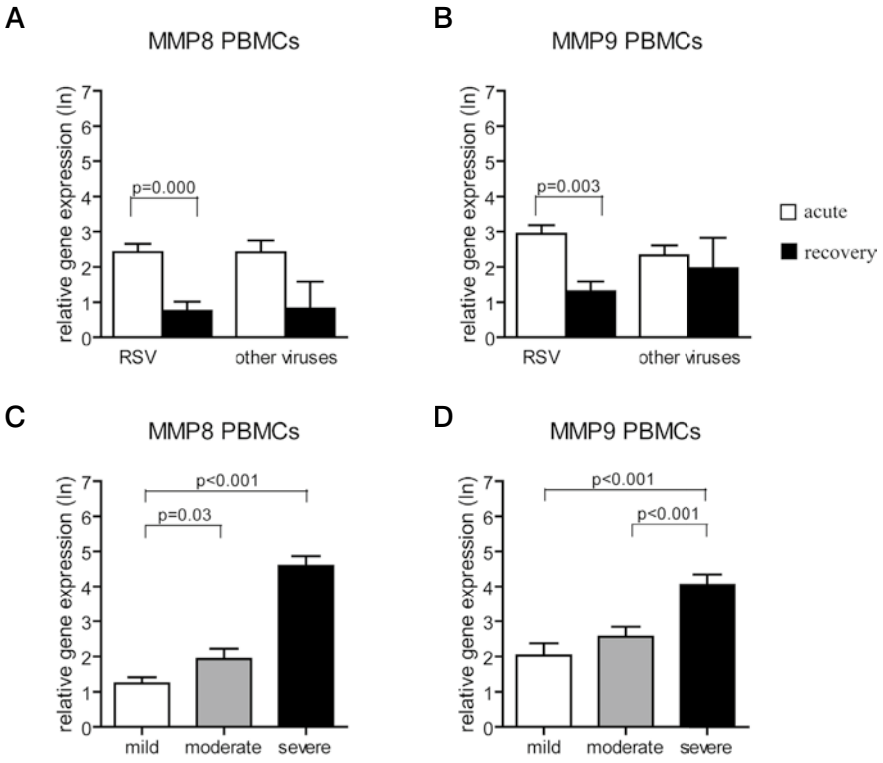

Figure 8.2 Gene expression levels of MMP-8 and MMP-9 in granulocytes from children with viral lower respiratory tract infections. Relative gene expression levels (mean ± standard error) of MMP-8 (**A**) and MMP-9 (**B**) in PBMCs from children during acute RSV positive and RSV negative viral lower respiratory tract infections and after recovery of infection. Relative gene expression levels of MMP-8 (**C**) and MMP-9 (**D**) for RSV positive children with mild, moderate, and severe disease. Mann–Whitney *U*-test was performed to compare children infected by RSV and other viruses. Paired analyses (Wilcoxon) were performed to compare acute and recovery samples. A two-sided value of $P < 0.05$ was considered statistically significant.

of MMP-9 expression was observed (**Figure 8.5B**). Unstimulated neutrophils secreted high levels of MMP-8 and MMP-9. Stimulation with LPS and RSV had no effect on the release of MMP-8 and MMP-9 by neutrophils (**Figure 8.5C**).

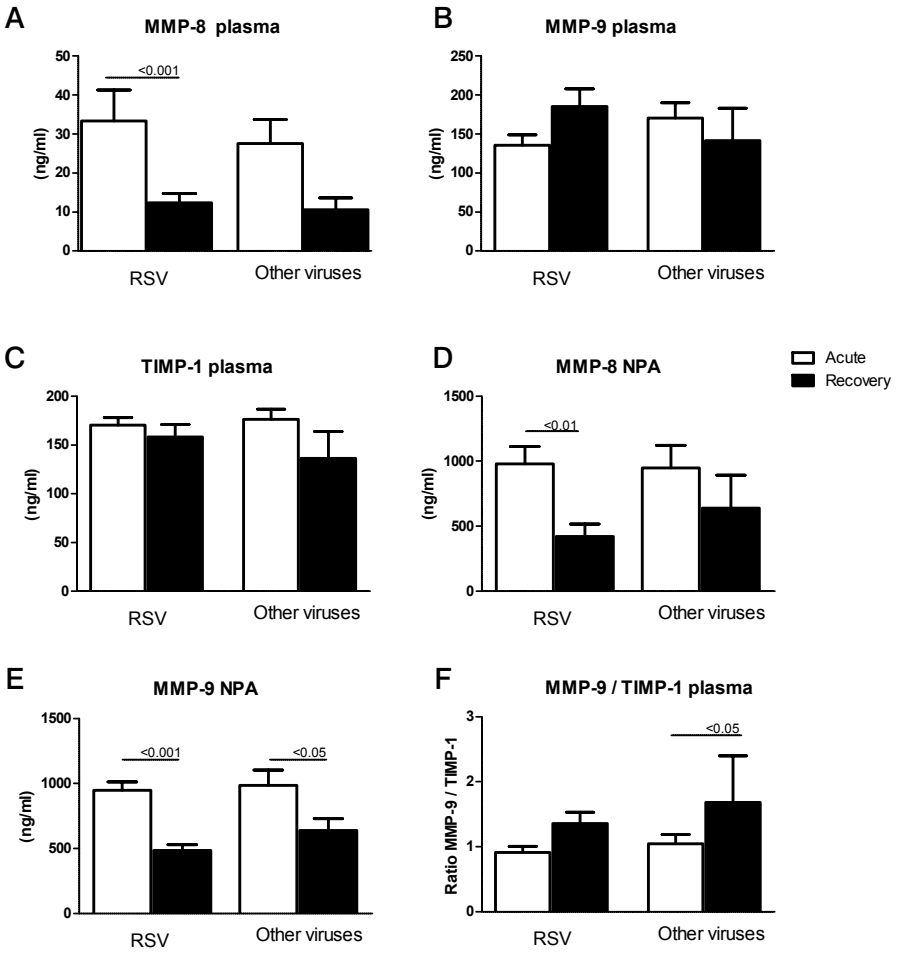

Figure 8.3 MMP-8 and MMP-9 concentrations in plasma and nasopharyngeal samples from children during acute lower respiratory tract infections and after recovery. Plasma concentration of MMP-8 (**A**), MMP-9 (**B**), TIMP-1 (**C**), and concentration in nasopharyngeal washes of MMP-8 (**D**) and MMP-9 (**E**) from children during acute RSV positive and RSV negative viral lower respiratory tract infections and after recovery of infection. Ratio's between plasma MMP-9 and TIMP-1 (**F**). Concentrations (ng/ml) are given in mean ± standard error. Mann–Whitney *U*-test was performed to compare children infected by RSV and other viruses. Paired analyses (Wilcoxon) were performed to compare acute and recovery samples. A two-sided value of $P < 0.05$ was considered statistically significant.

Discussion

This study demonstrates that disease severity of viral lower respiratory tract infections in children is associated with increased gene expression levels of the MMP-8 and MMP-9 genes in both PBMCs and granulocytes. These associations were observed in children with lower respiratory tract infections caused by either RSV or other respiratory viruses. The in vitro experiments in this study show that MMP-8 and MMP-9 mRNA and protein expression in PBMCs and granulocytes is not induced by stimulation with RSV. Consequently, other factors than direct viral interaction induce gene expression in PBMCs and granulocytes.

This is the first study that describes an association between MMP-8 and MMP-9 gene expression and disease severity of viral lower respiratory infections in children. This association was significant for children with a RSV infection and there was a trend for children with a viral lower respiratory infection caused by other viruses, indicating to a more general marker for disease severity during respiratory viral infections.

Several studies have shown that transcriptional analysis of peripheral blood cells can be used to discriminate the etiology and disease outcome.[29-32] Ramilo et al. compared the transcriptional profiles of PBMCs of children with infectious diseases, and identified a set of genes that could separate influenza A infections from bacterial infections (*Staphylococcus aureus*, *Escherichia coli*, and *Streptococcus pneumoniae*).[31] Both MMP-8 and MMP-9 were not represented in the selected set of classifier genes. Retrospective analysis of the microarray data set was performed by us, and showed that mRNA expression of MMP-8 and MMP-9 were elevated in all groups compared to controls, indicating a more general marker for inflammatory disease (data not shown). No data were available on disease severity, so it is not possible to exclude an association with disease severity. In an experimental model of viral infection of the upper respiratory tract in adults with RSV, influenza, and RV, no up- regulation of MMP-8 and MMP-9 was detected in whole blood transcriptional profiles.[33] However, these infections were all mild with consequently low levels of inflammatory markers.

This study shows that in MMP-8 plasma concentrations were increased during acute infection with RSV. Although MMP-8 plasma concentrations were higher in moderate and severe disease compared to mild disease, there was no step-wise relation with disease severity. This is in contrast with the gene expression data and indicates a different source of plasmaproteins than the circulating cell population. This is in line with the study of Hartog et al. in which they found elevated MMP-8 concentrations in plasma and lung fluid in adults with hospital-acquired bacterial pneumonia compared to healthy controls.[16] They found an association between clinical severity scores and MMP-8 concentrations in BAL fluid, but not in plasma.

Although MMP-9 plasma concentrations were increased during acute viral respiratory infections in children, no association between MMP-9 plasma concentrations and disease severity was found in this study. Previous studies have described such an association for several inflammatory diseases, such as pneumonia[16], tuberculosis infections[34], septic

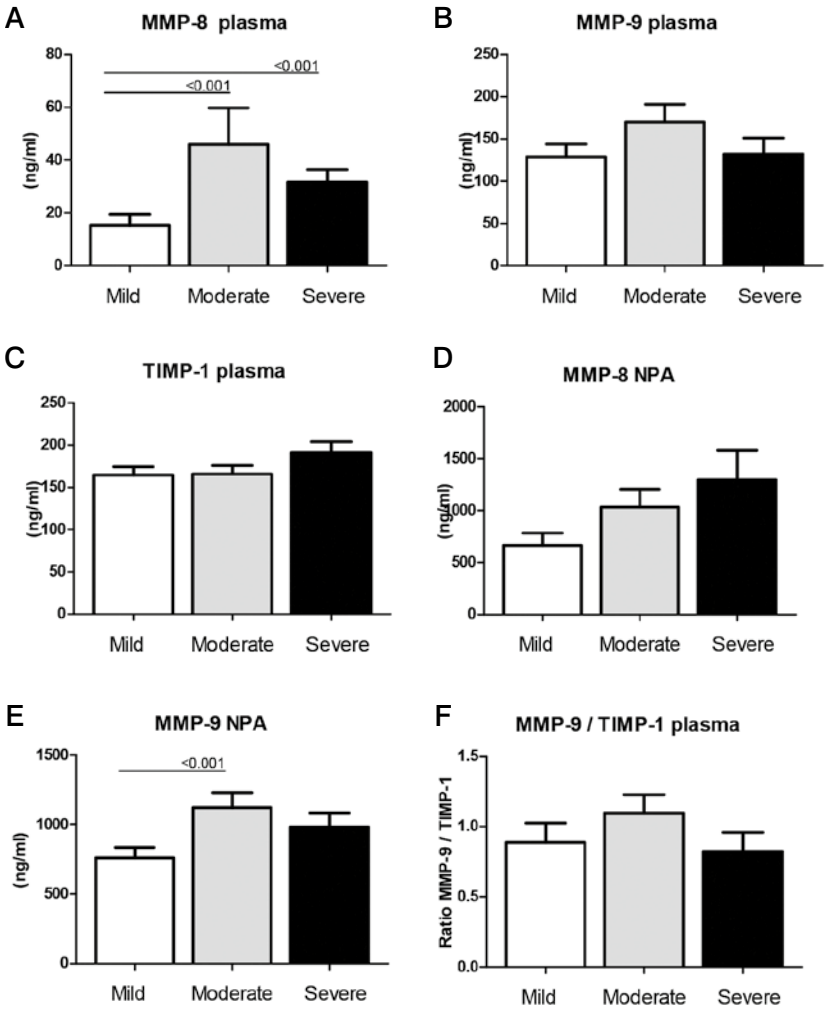

Figure 8.4 MMP-8 and MMP-9 concentrations in plasma and nasopharyngeal samples from children with mild, moderate, and severe RSV infection. For RSV positive children plasma concentration of MMP-8 (**A**), MMP-9 (**B**), TIMP-1 (**C**), and concentration in nasopharyngeal washes of MMP-8 (**D**) and MMP-9 (**E**) in mild, moderate, and severe disease are given. Ratio's between plasma MMP-9 and TIMP-1 (**F**). Concentrations (ng/ml) are given in mean ± standard error. Mann–Whitney *U*-tests were performed to compare mild, moderate, and severe disease. A two-sided value of $P < 0.05$ was considered statistically significant.

shock [35], and asthma[22]. This may be related to the fact that, in consistent with other studies [36-38], MMP-9 concentrations were correlated to neutrophil counts, although no significant differences in neutrophil counts between the different severity groups were observed in this study.

TIMP-1 is an inhibitor of the protease activity of all known MMPs.[39] Previous studies have described an association between an imbalance between MMP-9 and TIMP-1 and tissue degradation and airflow obstruction in asthma and chronic bronchitis.[37,40] In addition, elevated MMP-9/TIMP-1 ratios have been observed in plasma from patients with status asthmatics.[22] Furthermore, it has been shown that increased TIMP-1 concentrations, but not MMP-9, in nasopharyngeal washes of RSV- infected children correlated with disease severity and this suggests that a disturbed MMP-9/TIMP-1 homeostasis contributes to disease severity.[26] The ratio of MMP-9 and TIMP-1 concentration in plasma did not show a correlation with disease severity in this study and indicates that MMP-9 is differentially regulated at the mucosal level during infection.

Although both MMP-8 and MMP-9 concentrations in nasopharyngeal samples were increased during acute infection compared to recovery samples no association with disease severity was observed. The wide range of nasopharyngeal concentrations between individuals is partly due to the variation induced by aspiration volumes from the nasopharyngeal cavity. Currently, more standardized methods have been developed, such as flocked swabs, which can be used for viral diagnostics as well as protein analysis.[41,42] Normalization of protein levels to stable metabolites present in the mucus might further improve the use of nasopharyngeal samples for diagnostics, although these methods are not available yet. Further it should be taken in account that upper respiratory samples do not necessarily represent the situation in the lower airways and the systemic inflammatory response.

The in vitro experiments in this study show that MMP-8 and MMP-9 mRNA and protein expression in PBMCs and granulocytes were not induced by stimulation with RSV. Other factors than direct interaction between RSV and host cells could explain the increased gene expression levels of MMP-8 and MMP-9 in children with viral lower respiratory tract infections. Influx of bone marrow-derived neutrophil precursors in blood from children with severe RSV infections can result in higher MMP-9 expression due to granule protein production, such as MMP-8 and MMP-9, during immature stages of neutrophil development.[43] Also inflammatory mediators, such as growth factors, pro-inflammatory cytokines, oxidative stress upon viral infection can induce elevated gene expression levels of MMPs.[14] It has also been shown that the lung injury caused by mechanical ventilation has resulted in increased MMP-8 and MMP-9 expression.[44] However, in this study, the last mentioned cannot completely explain the differences in gene expression since also differences in gene expression between patients with mild and moderate disease were observed, all non-ventilated patients.

The results of this study indicate that neutrophils are the major source of MMP-9 production. The higher MMP-8 and MMP-9 concentrations in nasopharyngeal samples compared to plasma may therefore reflect the influx and degranulation of neutrophils in the airways during infection.

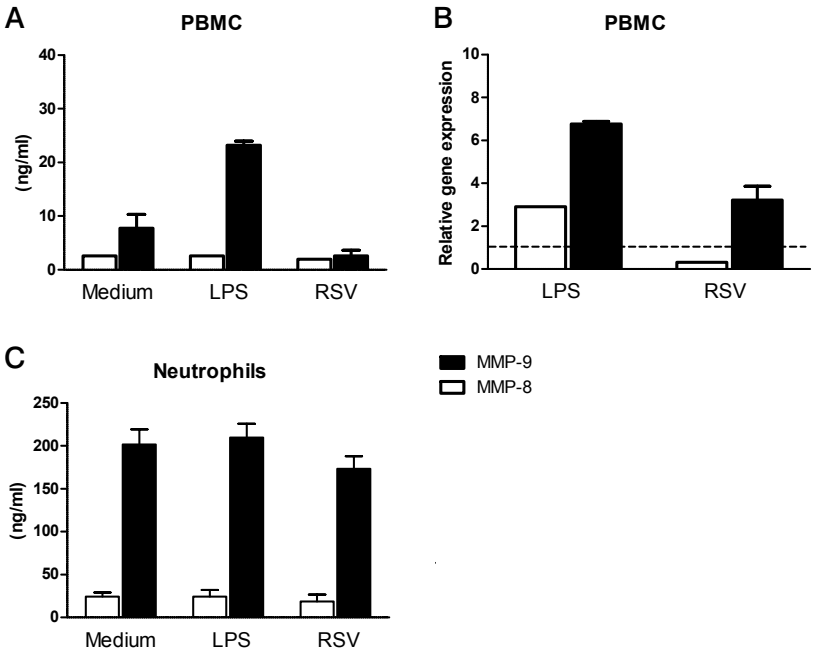

Figure 8.5 Stimulation of PBMCs and neutrophils by LPS and RSV in vitro. Human PBMC of healthy volunteers (n = 4) were stimulated with LPS (1 ng/ml) or RSV A2 (MOI 1) and MMP-8 and MMP-9 concentrations in supernatant were measured after 24 by ELISA (**A**) or at transcriptional level by q-PCR (**B**). Neutrophils were stimulated for 4 hr and MMP levels were determined in the supernatant (**C**).

This is in contrast to observations made by others that suggest that airway epithelial cells are the primary source of MMPs. It has been shown that MMP-9 gene expression is increased in human airway epithelial cell lines infected with RSV.[24] However, another study indicated that infected human airway epithelial cells are not the primary source of MMPs and TIMP-1 and that infiltrating leukocytes are responsible for MMP-9 in airway samples.[26] Also in a RSV infection model in mice, of which it is known that the epithelial cells are not infected, it was demonstrated that gene expression of MMP-9 is elevated in the lungs most likely by infiltrating cells.[25] For MMP-8, no correlation with neutrophil counts was observed and gene expression levels in granulocytes and PBMCs were comparable indicating that MMP-8 transcription and secretion was different regulated than MMP-9. This is supported by differences in the degranulation of subcellular neutrophilic granules, in which MMP-8 and MMP-9 are stored and differences in transcriptional events that induce MMP-8 and MMP-9 mRNA expression.[45] For example, it has been shown that pro-inflammatory cytokines, particularly IL-1b, play a central role

in the modulation of MMP-8 expression.[46,47] Future studies may reveal the role for MMP-8 plasmaconcentrations as a potential biomarker to assess disease severity in viral lower respiratory tract infections in children.

The relation of MMP-8 and MMP-9 with viral load in the nasopharyngeal cavity have not been investigated in this study. In experimental respiratory viral infection models, inflammatory markers such as cytokines correlated with viral load and the symptom scores, indicating that the amount of virus is the driving force for inflammation.[48] Also in children, disease severity has been associated with high viral titers.[49,50] Further research might reveal the role of inflammatory mediators in the pathogeneses of severe respiratory viral infections.[51] In this light, it is interesting to consider inflammatory mediators, such as MMPs, as potential targets for therapy.

Some limitations of this study need to be considered. First, the younger age of the children with the most severe lower respiratory tract infections may have caused a bias in the results. However, age was not correlated with MMP-8 and MMP-9 plasma concentrations, which suggests that age alone cannot explain the observed differences. According to these results, did not found significant differences in MMP-8 and MMP-9 serum concentrations in children, 2–18 years of age.[52] Second, multiple viruses were detected in 40% children with lower respiratory tract infections and it cannot be entirely exclude that the presence of multiple viruses have induced a different inflammatory response.

The results of this study suggest that the expression of MMP-8 and MMP-9 genes are potential marker candidates for diagnostic use to assess disease severity in children with viral lower respiratory tract infections. Markers for disease severity do not have clinical implication at present, because currently antiviral treatment is not available. However, new antiviral treatment of patients will be based most likely on diagnostics predicting disease severity and susceptibility.[53,54] Furthermore, markers for disease severity are also important for research purposes to study effects of interventions, such as treatment. Although currently no biomarkers at transcription level are available in the clinic, because the processing is time consuming, innovative techniques may enable rapid analysis of the expression of multiple genes at transcriptional level in the near future.[55]

In conclusion, increased expression of the MMP-8 and MMP-9 genes was observed in PBMCs and granulocytes obtained from children with severe viral lower respiratory tract infections. MMP-8 and MMP-9 gene expression levels in circulating cells may be useful markers to support clinical evaluation of disease severity in viral respiratory infections. These results justify future follow-up, that is, the validation in independent prospective study cohorts of the usefulness of MMP-8 and MMP-9 as a potential markers for disease severity in viral respiratory infections.

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9

Olfactomedin 4 serves as a marker for disease severity in pediatric Respiratory Syncytial Virus (RSV) infection

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Abstract

Background: Respiratory viral infections follow an unpredictable clinical course in young children ranging from a common cold to respiratory failure. The transition from mild to severe disease occurs rapidly and is difficult to predict. The pathophysiology underlying disease severity has remained elusive. There is an urgent need to better understand the immune response in this disease to come up with biomarkers that may aid clinical decision making.

Methods: In a prospective study, flow cytometric and genome-wide gene expression analyses were performed on blood samples of 26 children with a diagnosis of severe, moderate or mild Respiratory Syncytial Virus (RSV) infection. Differentially expressed genes were validated using Q-PCR in a second cohort of 80 children during three consecutive winter seasons. FACS analyses were also performed in the second cohort and on recovery samples of severe cases in the first cohort.

Results: Severe RSV infection was associated with a transient but marked decrease in CD4+ T, CD8+ T, and NK cells in peripheral blood. Gene expression analyses in both cohorts identified Olfactomedin4 (OLFM4) as a fully discriminative marker between children with mild and severe RSV infection, giving a PAM cross-validation error of 0%. Patients with an OLFM4 gene expression level above -7.5 were 6 times more likely to develop severe disease, after correction for age at hospitalization and gestational age.

Conclusion: By combining genome-wide expression profiling of blood cell subsets with clinically well- annotated samples, OLFM4 was identified as a biomarker for severity of pediatric RSV infection.

Introduction

Respiratory viral infections are an important cause of hospitalization among children younger than 5 years of age. Human Respiratory Syncytial Virus (RSV) is the most common (40–85%) identified virus in infants hospitalized for respiratory infections during winter epidemics, with hospitalization rates between 1 and 2%.[1-6] Clinical manifestations range from common colds to severe lower respiratory tract infections requiring mechanical ventilation. Risk factors for a severe course are known, but the majority of patients admitted to an Intensive Care Unit were previously healthy. [7-9] Since transition from mild to severe disease can occur within hours, one of the key challenges for clinicians is to differentiate children who need hospitalization for supportive care from those who can safely be discharged. Currently, young infants with mild bronchiolitis, especially those younger than 12 weeks of age, are often admitted to a hospital since they have an increased risk of severe disease. However, up to 35% of children hospitalized with bronchiolitis do not receive any supportive intervention.[10] Conversely, it is crucial to avoid early discharge of those children who may experience clinical deterioration. Among children sent home with the diagnosis bronchiolitis, 4.6–6.8% require hospitalization later on.[11,12]

Much research has been done on the immune response against RSV in humans. Several reports suggested an important role for the innate immune system, while others found an inadequate adaptive immune response especially in young children and in individuals who present with a severe clinical picture.[13] The uncertainty in the nature of the immune response against RSV is reflected in the unpredictable clinical course of the infection as well as in the difficulty of developing an adequate vaccine. We as well as others previously reported that T lymphocytes can be markedly decreased in the more severe cases of the disease. We reported that in severe cases both CD4 and CD8 T cell numbers, as well as NK cells were reduced in peripheral blood.[14] However, it remains unclear whether this indicates an inadequate immune response against RSV, for instance by massive apoptosis or decreased production of T cells, or that peripheral blood poorly reflects an ongoing immune response that might be very active.

The detection and application of biomarkers to assess severity of viral lower respiratory tract infections, in particular RSV infection, may assist clinicians in the prediction of severe disease in children with bronchiolitis and may help to reduce the number of unnecessary hospitalizations or clinical deterioration after discharge. Furthermore, markers for disease severity are important research tools to study effects of interventions by new therapies or to stratify patients by disease severity.[15,16] Several studies have shown that transcriptional analysis of peripheral blood cells may be used to define different etiologies of disease and disease outcomes.[17-20] In a seminal proof-of-concept study, Ramilo *et al*. (2007) compared the transcriptional profiles of PBMCs of children with infectious diseases and identified a set of genes that can separate influenza A infections from bacterial infections (*Staphylococcus aureus*, *Escherichia coli* and *Streptococcus pneumoniae*).[20] Recently this group also reported that these transcriptome

profiles also contained information regarding viral etiology (influenza, rhino virus and RSV) and the course of disease.[21]
This study was initiated to obtain insight into the changes occurring in adaptive and innate immune cells during RSV infection and to identify possible biomarkers of disease severity. To identify transcriptional biomarkers to separate mild from severe disease, genome-wide gene expression analyses were performed on blood samples of 26 children with a diagnosis of severe, moderate or mild RSV infection in two winter seasons. A validation cohort of 80 children spanning three other consecutive winter seasons by flow cytometry and Q-PCR was used to validate various candidate biomarkers.

Material and Methods

Study design

In this prospective cohort study, 3 ml of Sodiumheperanized blood and nasopharyngeal samples were obtained from two cohorts of patients with RSV bronchiolitis within 24 hours after first contact with the hospital. Medical history, demographic data, and clinical assessments were collected from questionnaires and medical records. Exclusion criteria were corticosteroid use in past 48 hours, congenital significant heart or lung disease and immunodeficiency, Presence of 15 different viral pathogens was tested by multiplex RT-PCR on nasopharyngeal samples as previously described.[22] Patients were classified retrospectively into three groups based on severity of disease. The mild group included children without hypoxia or severe feeding problems. The moderate group included children requiring hospitalization for supplemental oxygen (oxygen saturations <93%) and/or nasogastric feeding. Children requiring mechanical ventilation were included in the severe group. Recovery samples were obtained 4–6 weeks after acute infection from children with moderate and severe disease. The first cohort consisted of 26 patients with RSV infections, divided into mild (n = 9), moderate (n = 9) and severe (n = 8) disease. From all moderate and severe diseased patients recovery samples were obtained. This cohort was used for micro-array analysis and initial qPCR validation of genes of interest. The second cohort comprised 80 children with viral lower respiratory tract infections both RSV positive and negative, and was also divided into three groups: mild (n = 14), moderate (n = 42) and severe (n = 24). This cohort was meant for validation purposes. All subjects were recruited at two hospitals in Nijmegen, the Canisius Wilhelmina Hospital and the Radboud university medical center, the Netherlands. The study protocols were approved by the institutional review board (Commissie Mensgebonden Onderzoek: Regional Committee on Research involving Human Subjects Arnhem-Nijmegen, The Netherlands) and were conducted in accordance with the principles of the Declaration of Helsinki. Written informed consent was obtained from the parents of all children.

RNA isolation and microarray gene expression analyses

Peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation (Lymphoprep, Axis Shield, Norway), counted and subsequently stored in Trizol reagent (Invitrogen, The Netherlands) at -80°C in the same laboratory by the same team for both cohorts. RNA from PBMC was extracted using Trizol (Invitrogen Life Technologies) according to the manufacturer's protocol. Total RNA was isolated using the RNeasy Minikit (Qiagen). RNA integrity and quality was assessed using capillary electrophoresis [RNA 6000 Nano LabChip (Agilent)] on an Agilent Bioanalyzer 2100 system. RNA processing, target labeling and hybridization to gene expression arrays was performed by standard methods as described.[23] Biotin labeled cRNA was obtained using the One-Cycle Eukaryotic Target Labeling Assay Affymetrix), after which 15 μg of fragmented, biotin labeled cRNA was hybridized to Affymetrix GeneChip Human Genome U133 plus 2.0 arrays according to standard Affymetrix protocol (Affymetrix Inc, Santa Clara, CA).

Flow Cytometry

Immunophenotyping of cryopreserved PBMCs were performed after thawing. The following combinations of markers and fluorescent antibodies were used: CD14–FITC, CD16.56–phyco- erythrin, CD3–peridinin chlorophyll protein, CD19–allophycocyanin, CD4–phycoerythrin– Cy7, and CD8–allophycocyanin–Cy7 (all Beckman Coulter, Miami, FL). Samples were acquired immediately after staining on a BD FACSCanto (Becton Dickinson, Heidelberg, Germany) and analyzed using flow cytometry analysis software (FlowJo analyses 7.6, Three Star, Ashland, OR). The following subsets were defined: CD4+ T cells (CD4+CD3+CD8-), CD8 T cells (CD8+CD4-CD3+), NK cells (CD3-CD56+), B cells (CD45+CD19+) and monocytes (CD14+).

Data analysis

Quality control analyses were performed as previously described.[23,24] Scanned images were inspected for artifacts, percentage of calls present (<25%) and controls of RNA degradation. This led to some arrays being discarded. On each remaining array, probes labelled outliers by the Affymetrix scanning software and overexposed probes (with maximum PM intensity level >63.000) were removed. Subsequently, probesets with less than 8 probes remaining were discarded. For each comparison, robust multichip analysis (RMA) was used for background removal, quantile normalization of probe intensity levels and probe set summarization. The resulting values were log2-transformed for further analysis, giving probeset expression levels between 0 and 16. We then selected only those probesets that showed at least a two-fold difference (up or down) on a minimum of two arrays with respect to the median expression over all arrays in that particular comparison.[24,25] Finally, Significance Analysis of Microarrays (SAM)[26] was applied to find differentially expressed probesets with a significance level of q <0.05. To select only biologically relevant changes, we demanded additionally that the absolute expression level was larger than log2(200) and that the absolute difference between groups was larger than 2 fold.

In a subsequent supervised analysis, we trained a PAM classifier ("Prediction Analysis of Microarrays"[27], attempting to find a minimum number of discriminative genes that yielded an optimal cross-validation error (i.e. the predicted test error). For visualization purposes, samples were clustered based on selected probesets by complete linkage hierarchical clustering with 1-correlation as a distance measure, using the Matlab Bioinformatics toolbox (Mathworks, Natick, MA). The original and processed data were deposited in the NCBI Gene Expression Omnibus (GEO; http:/ /www.ncbi.nlm.nih.gov/ge o ; GSE69606). All microarray experiments were performed according to the MIAME guidelines.

RT-PCRReal-time quantitative PCR was used to measure the expression of genes of interest. Initial validation of gene expression of OLFM4 detected in the first cohort was performed with SYBR Green PCR Mastermix (Applied Biosystems; P/N 4367659) with forward 5`- atcaaaacacccctgtcgtc- 3`and reverse 5`- gctgatgttcaccacaccac-3`primers for OLFM4. Actin was used as a reference gene with forward primer 5`- cgtcacacttcat-gatggagttg-3`and reverse primer 5`- cttccttcctgggcatgga-3`. After validation of the microarray, the second cohort was analyzed with commercially available Taqman primers (OLFM4 Hs00360669_m1 and GAPDH Hs99999905_m1). All samples were run for 40 cycles in duplicate on an Applied Biosystems 7500 Fast Real-Time PCR System. Ct values of OLFM4 were normalized against the reference gene GAPDH.

OLFM4 plasma measurement

OLFM4 concentrations were measured in randomly selected plasma samples of 49 patients from the validation cohort by a commercial ELISA kit (E90162Hu, Uscn Live Science Inc., China) according to the instructions of the manufacturer.

Published microarray data mining

A data mining search was performed in NCBI GEO and in EBI Arrayexpress, online databases with datasets and profiles of previously performed microarray studies to validate our results.[28,29] Terms for searching were: *OLFM4*, *Affymetrix*, *whole blood children*, *RSV and/or homo sapiens*. More than 90 microarray studies were found. Based on the population (children/infants), sample size, disease type and available information per sample, 18 studies were selected. From the series matrix files, the results were log transformed and OLFM4 gene expression was selected and analyzed to gain insight in its behavior in different disease states and ages.

Statistics

The distributions of categorical variables are presented as percentages per category. Numerical variables are reported as means with standard deviation (SD) or medians with interquartile ranges (IQR) depending on whether or not the variables were normally distributed (Kolmogorov-Smirnov's test, $p>0.05$). To determine whether OLMF4 was independently associated with receiving mechanical ventilation, multivariable log-binomial regression analyses were per- formed in the validation cohort resulting in adjusted Relative Risks (RR).[30] Analysis were performed with SPSS v21 and graphpad v5.

Results

We previously reported that RSV infection, especially in severe cases, was associated with lymphopenia. This was not only visible in NK and CD8+ T cells, known to be directly involved in anti-viral immunity, but surprisingly also in CD4+ T cells, whereas B cells were unaffected.[14] In the current study, we analyzed recovery samples of 6 severe patients after clearance of the infection (on average 4 weeks after discharge) and found that the numbers of NK cells as well as CD4+ and CD8+ T cells had returned to normal, indicating that the lymphopenia was transient (S1 Fig).

Microarray analyses point to OLFM4 as a marker gene to classify disease severity

Patients used for microarray analysis with a clinical diagnosis of mild disease were older at time of admission to hospital than those with severe disease. The length of stay in hospital increased with increasing disease severity. No statistically significant differences were seen in gender, number of premature infants and duration of symptoms (**Table 9.1**). The microarray analysis of PBMC of children with mild versus severe disease showed that 564 probesets were expressed differentially (428 upregulated and 136 downregulated genes) under conditions as described in material and methods (q <0.05; >2 fold difference; absolute expression value > log2(200). As biomarkers should discriminate between non-disease and disease, the genes expressed differentially in children with mild versus severe disease as well as during acute severe RSV infection versus recovery were selected.

Table 9.1 Patient characteristics.

	Mild (N = 9)	Moderate (N = 9)	Severe (N = 8)	p-value
Age (months)	8.7 [3.6–9.3]	1.9 [1.5–8.3]	2.4 [1.1–4.9]	NS
Gender (male)	6 (67%)	8 (89%)	6 (75%)	NS
Gestational age (wks)	40 [36.9–41.0]	38.6 [37.2–40.0]	35.1 [33.1–39.8]	NS
Length of stay (days)	0 [0–3]	5 [2–9]	13 [6.3–19.8]	$p<0.001$*

Values are given in numbers (percentages) and median with inter quartile range (IQR). *P*-values are based on Kruskall Wallis tests, followed by Mann Whitney U tests for individual comparisons.

*mild vs moderate $p<0.01$, moderate vs severe $p<0.05$, mild vs severe $p<0.001$

The analysis of paired acute and recovery samples of children with severe RSV infection resulted in 808 differentially expressed probesets (647 upregulated and 161 downregulated). Of these 808 probesets, 448 showed over- lap with the 564 probesets in the comparison of mild versus severe disease, 365 genes being upregulated and 83 genes being downregulated (**Figure 9.1 and Figure S2**). **Table 9.2** shows the top 25

of up- and downregulated genes, of which *Olfactomedin 4* (OLFM4) was the most upregulated gene with a factor of over 40 fold. Since children in the severe group were younger compared to those with mild disease, a paired age-matched subanalysis was performed among 7 severe patients versus 7 patients with mild or moderate disease. This analysis resulted in 287 differentially expressed probesets, all upregulated. The gene list of upregulated probesets did not differ substantially from the main analysis. A supervised analysis (PAM) also identified OLFM4 as a fully discriminative marker between children with mild and severe RSV infection, giving a cross-validation error of 0%. As both SAM and PAM analyses revealed OLFM4 as a potentially important marker for disease severity in children with RSV infection and OLFM4 has-to the best of our knowledge- not been associated with respiratory tract infections before, this gene was chosen for further analysis. Interestingly, there was no marked upregulation of apoptosis genes in the severe group, indicating that the observed lymphopenia was not caused by increased apoptosis.

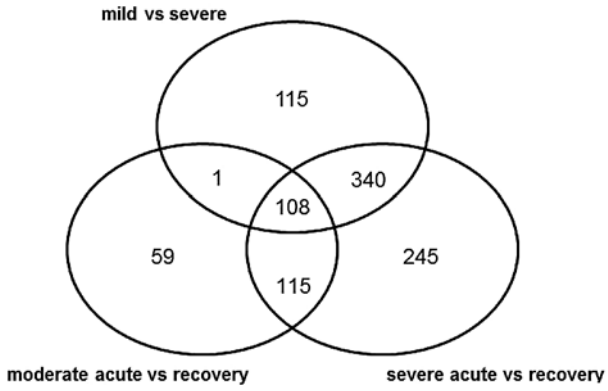


Figure 9.1 Venn diagram with differentially expressed genes between groups. Differentially expressed genes (q <0.05; >2 fold difference; absolute expression value >200) in patients with RSV infections comparing patients with mild vs severe disease and during acute infection vs recovery in patients with moderate and severe disease.

Validation of microarray findings by qPCR

To confirm our findings in the microarray analyses, qPCR was performed for OLFM4 in PBMCs of the same patients. One patient in the moderate group was excluded since insufficient material was left. OLFM4 expression was statistically significantly higher in patients with severe disease compared to those with mild and moderate disease (**Figure 9.2A**).

Table 9.2 Top 25 up- and downregulated genes differentially expressed in PBMCs during severe RSV infection.

Upregulated genes	Fold difference mild vs severe	Fold change severe vs recovery	Downregulated genes	Fold difference mild vs severe	Fold change severe vs recovery
OLFM4	49.7	43.2	**GNLY**	-4.8	-4.0
MMP8	24.2	37.2	**GZMH**	-4.5	-3.2
MMP8	24.1	33.6	**GNLY**	-4.5	-3.7
CAECAM8	18.4	19.3	**FGFBR2**	-4.5	-5.1
ARG1	16.9	24.2	**TRAC**	-3.9	-2.9
ANXA3	16.6	16.2	**KLRF1**	-3.8	-3.3
DEFA4	15.1	16.1	**LGALS2**	-3.7	-2.0
CA1	14.9	17.5	**KLRC1**	-3.7	-3.2
CHI3L1	13.9	11.0	**KLRD1**	-3.7	-3.0
LTF	13.0	12.6	**TRAC**	-3.4	-2.8
SELENBP1	12.8	15.8	**THOC4**	-3.4	-3.1
CRISP3	12.3	12.3	**GZMB**	-3.4	-2.3
ELANE	11.2	10.6	**KLRD1**	-3.4	-2.7
HP	11.0	11.9	**IGHM**	-3.3	-2.0
CEACAM6	11.0	10.1	**GZMK**	-3.3	-2.4
HBM	10.5	26.0	**PRF1**	-3.2	-2.2
CHI3L1	10.3	9.2	**TRAC**	-3.1	-2.3
MPO	10.2	9.9	**ITPKB**	-3.0	-3.5
ALAS2	10.2	21.7	**SH2D1B**	-3.0	-2.6
IL1R2	10.2	10.5	**SPON2**	-3.0	-2.8
EPB42	10.1	15.3	**PRF1**	-3.0	-2.4
CEACAM6	10.1	9.7	**TGFBR3**	-2.9	-2.4
LCN2	10.1	10.3	**FCER1A**	-2.9	-3.9
MPO	9.8	9.9	**KLRB1**	-2.9	-3.9
MMP9	9.8	11.6	**GPR56**	-2.9	-2.9

Genes that showed overlap in both the comparison mild vs severe disease and severe vs recovery samples are shown.

OLFM4 gene expression in PBMCs is increased during acute viral respiratory infection and correlates with disease severity in a validation cohort

The validation cohort consisted of 80 children with viral lower respiratory tract infections, among which 47 had a confirmed RSV infection. This cohort reflects the patients presenting in a paediatric ward during respiratory season prior to viral diagnostics, therefore both

RSV positive and negative patients were analysed. The characteristics of these patients differed from those of the patients in the discovery cohort, especially for age, gender, preterm birth, duration of symptoms. (S1 Table). In total, 115 PBMC samples were available for qPCR analysis, subdivided in 80 acute and 35 recovery samples. OLFM4 gene expression levels during acute infection were higher compared to those obtained after recovery ($p<0.001$). In agreement with our microarray analyses, expression of OLFM4 in PBMCs was higher in patients with severe disease compared to those with mild and moderate disease (**Figure 9.2B**). For the confirmed RSV positive patients only,

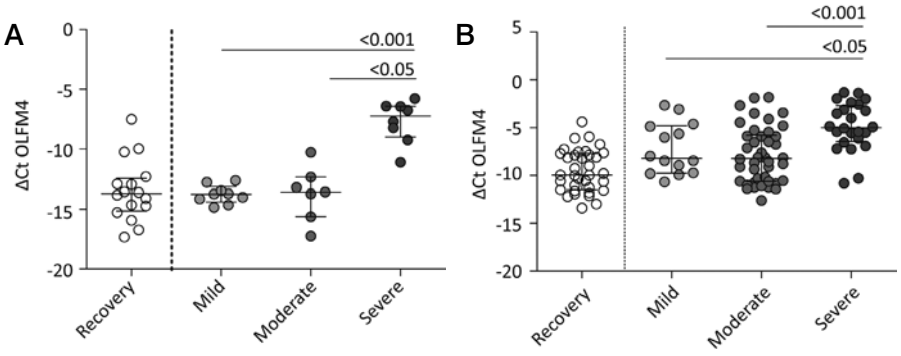


Figure 9.2 OLFM4 gene expression values of patients from the micro-array and validation cohort. OLFM4 gene expression levels were significantly higher in patients with severe disease compared to those with mild and moderate disease in the micro-array cohort (Panel A) and an independent validation cohort (Panel B). Expression levels are presented as ΔCt and median with inter quartile range (IQR). Statistics were performed by Kruskall Wallis tests, followed by Mann Whitney U tests for individual comparisons.

OLFM4 expression also served as a discriminating marker, similar to the full validation cohort (S3 Fig). Length of stay (LOS) in hospital, another measure for severity, was also correlated positively with gene expression levels of OLFM4 ($\rho = 0.402$, $p<0.001$).

OLFM4 expression correlates with disease severity in PBMCs but not in plasma

Since biomarkers in plasma are more easily obtained and processing is less time-consuming, we measured protein levels of OLFM4 in plasma of 49 randomly selected patients of the validation cohort. Although OLFM4 plasma concentrations during acute infection were statistically significantly higher compared to those in recovery samples, no association with disease severity was observed (**Figure 9.3**). No correlation between protein levels and relative gene expression was found either ($\rho = 0.270$, $p = 0.088$).

In a multivariable model OLFM4 gene expression is a statistically significant marker for severe disease

To determine the predictive value of OLFM4 in patient with acute viral bronchiolitis, both RSV positive and negative patients we performed a multivariable analysis. Relative OLMF4 gene expression, gender, gestational age, and age at time of hospital admission (in weeks) were included as determinant and potential confounders, respectively in a multivariable model for mechanical ventilation (**Table 9.3**).

For OLMF4, a cut-off value of > -7.5 was chosen, which corresponds with an OLFM4 expression level greater than 0.5% that of GAPDH. The unadjusted RR of mechanical ventilation was 8.6 with a 95% confidence interval (CI) of 2.2–34.0. After adjustment for age and gestational age, the RR was 6.1 (95%CI: 1.5–24.4), which indicates that children with OLMF4 gene expression levels above -7.5 have a 6-fold increased risk of severe infection requiring mechanical ventilation (**Table 9.4**). Gender did not add substantially to the final model. Including OLMF4 gene expression in the model as a continuous variable resulted in an age and gestational age adjusted RR of 1.20 (95%CI: 1.04–1.38), meaning that the risk of receiving mechanical ventilation increased by 20% with every step increase in expression level (range -12.6 through -1.33).

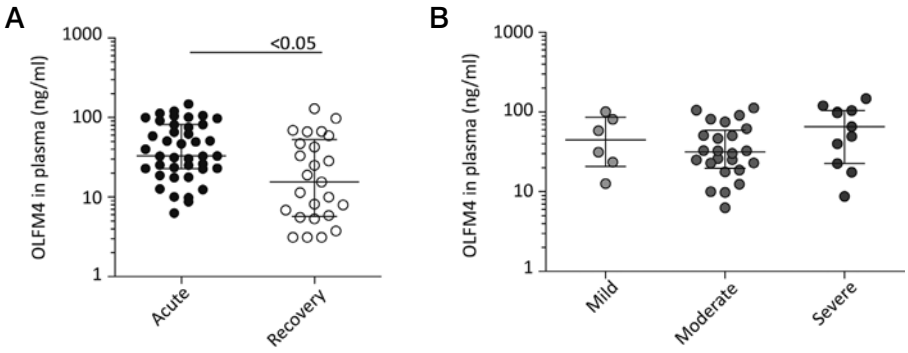


Figure 9.3 Plasma levels of OLFM4 in patients with viral RTI. OLFM4 plasma levels are statistically significantly higher during acute (n = 41) infections compared to recovery samples (n = 25) (Panel A). However, there are no statistically significant differences among the three severity groups (Panel B). Plasma levels (ng/ml) are presented as median with inter quartile range (IQR). Statistics were performed by Mann Whitney U tests for comparison acute vs recovery ($p<0.05$), and Kruskall Wallis tests for comparison mild vs moderate vs severe ($p = 0.29$).

Table 9.3 Multivariable analysis of the association between OLFM4 expression levels and the risk of getting mechanical ventilation.

	Characteristics			Univariate analysis (n=80)	Multivariate analysis		
	MV (n=24)	No MV (n = 56)	p-value	p-value	p-value	OR	(95% CI)
Gender (male)	12 (50%)	30 (54%)	NS	NS	-	-	-
Age (weeks)	1.22 [0.63–2.84]	3.95 [1.95–12.7]	<0.001	<0.01; OR 0.96	0.05	0.96	0.93–1.00
OLFM4 expression	-4.89 ± 2.53	-7.68 ±2.86	<0.001	4,71*10E-4; OR 1.43	<0.01	1.48	1.14–1.91
Premature (<37wks)	4 (17%)	11 (20%)	NS	NS	-	-	-
Duration symptoms	11.0 [10.0–13.0]	5.0 [2.0–4.0]	NS	NS	-	-	-

Values are given in numbers (percentages) and mean ± standard deviation. MV = mechanical ventilation, NS = not significant, OR = odds ratio, CI = confidence interval

Table 9.4 Relative risk of getting mechanical ventilation determent by OFFM4 gene expression.

	Multivariate analysis	p-value	OR 95% (CI)	Relative risk
OLFM4 expression> -7.5	<0.01	15.78	1.93–46.57	8.7

OR = odds ratio, CI = confidence interval

Discussion

In this study we demonstrated that OLFM4 gene expression in PBMC is a previously unidentified classifier for severe disease in children with viral lower respiratory infections. OLFM4 expression was significantly increased during acute viral respiratory infections compared to recovery samples. Moreover, an association was found between OLFM4 gene expression in PBMCs and disease severity; in a multivariable model OLFM4 showed its power as a significant marker for severe disease. Children with mechanical ventilation have almost 10 times more often an increased OLFM4 expression in PBMC (greater than 0.05% of the GAPDh levels).

Therefore, OLFM4 fulfills the criteria as a biomarker for disease severity, in particular to discriminate mild from severe cases in young infants. For OLFM4 to formally be used as prognostic marker, a more extensive, prospective study will be required.

To the best of our knowledge, the OLFM4 gene has not been described in the context of viral respiratory infections. Although changes in cell populations occur during the acute phase of infection could be reflected in the gene expression profiles, it is remarkable that predominantly upregulated genes indicate severe disease, whereas only decreased cell populations are observed.[14] To validate the OLFM4 gene expression changes during infectious disease we data-mined other micro-array studies that described pediatric and adult patient cohorts. In a study by Ioannidis *et al.* (GSE 34205), we found that OLFM4 gene expression was higher in PBMCs obtained from patients with RSV (n = 51) or influenza virus (n = 27) infections com- pared to the gene expression in healthy infants (n = 22), $p<0.01$ and $p<0.0001$, respectively.[31] No differences were found in OLFM4 gene expression between children under or above three months of age with either infection by RSV or influenza. In contrast, two other studies did not observe upregulation of OLFM4 in PBMCs from children during infection by measles or rotavirus (GSE 5808 and 2729).[32,33] Zaas *et al.* performed microarrays on whole blood obtained from adult volunteers at baseline and at the peak of their symptoms after being experimentally infected with RSV, influenza or rhinovirus (GSE 17156) .[34]Although their data showed an upregulation of OLFM4 in RSV infected adults ($p = 0.01$), there were no differences in OLFM4 gene expression between the baseline and during symptomatic influenza or rhinovirus infections.[34] Data of Ramilo *et al.* (GSE 6269–1) showed a statistically significant upregulation of OLFM4 in children, aged 0–16 years, diagnosed with influenza virus or bacterial infections (*E. coli*, *S. aureus* or *S. pneumoniae*) compared to healthy controls .[20] In this cohort, children with *S. aureus* or *S. pneumoniae* infections had statistically significantly higher OLFM4 gene expression compared to influenza A infected patients.[20] Thus, other studies have also seen upregulation of OLFM4 expresion after bacterial or viral infection. However, none of the studies looked at disease severity. OLFM4, also known as hGC-1 and GW112, was first cloned from G-CSF–stimulated human myeloid precursor cells and is mainly expressed in bone marrow, gastro-intestinal tract, prostate and pancreas.[35] Earlier studies have shown that OLFM4 is involved in multiple cellular functions e.g. cell growth, differentiation and apoptosis.[27] OLFM4 expression has been reported as one of several (prognostic) markers in oncology.[27] In addition, its involvement in the immune response to inflammation has been described. OLFM4 expression is upregulated in some inflammatory diseases, such as chronic inflammatory bowel diseases[36] and in *Helicobacter pylori*-infected patients .[37] Liu *et al.* showed an enhanced immune response and inflammation in OLFM4-/- mice upon *Helicobacter pylori* infection. Their results indicate that OLFM4 inhibits NOD1 and NOD2-mediated NF-κB activation, suggesting that OLFM4 plays an important role in regulating innate immune responses.[38] In another study, Liu *et al.* demonstrated that neutrophils from OLFM4 -/- mice have increased capability to kill *S. aureus* and *E. coli* and are more resistant to systemic sepsis .[39] These data suggest that OLFM4 may be an important regulator of host innate immunity against a broad array of bacterial infections. Data mining of gene expression profiling datasets (www.immgen.org) indicates that OLFM4 is besides being expressed in neutrophils, also highly expressed in Th1 cells.

Therefore the increased OLFM4 expression seen in the severe subgroup may indicate an highly active ongoing Th1 response. In summary, OLFM4 was upregulated in several viral and bacterial infections in many (but not all) previously published studies investigated.

Although OLFM4 mRNA has been described to be selectively expressed in normal human myeloid lineage cells, OLFM4 protein concentrations have been measured in PBMCs, B-lymphocytes, neutrophils and monocytes.[40] This is in agreement with our results and those from the reanalyzed microarray studies, in which high and significantly different OLFM4 mRNA expression was found in PBMCs obtained from children with different severity of viral lower respiratory infections.

Clemmensen *et al*. showed that OLFM4 was present at protein level in only 20–25% of peripheral blood neutrophils, whereas mRNA for OLFM4 was present in all myelocytes and metamyelocytes, indicating post-transcriptional regulation as a basis for the heterogeneous expression of OLFM4 protein.[41] This could explain the observed differences in our study between the transcription levels and the plasma concentration of the protein.

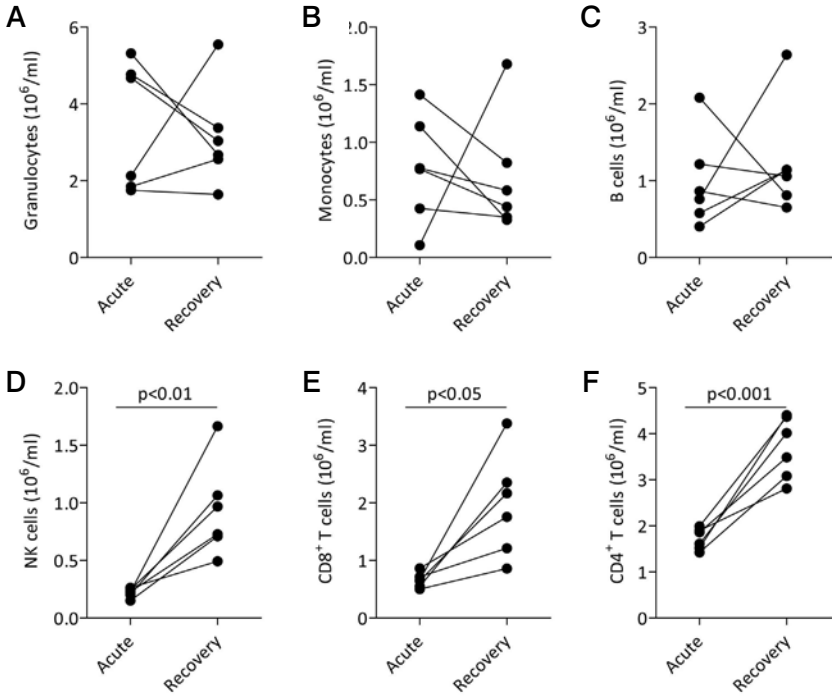
The advantage of measuring markers in plasma is the ease of implementation, speed, reproducibility and standardization. However, innovative techniques enable rapid analysis of the expression of multiple genes at transcriptional level in the near future.[42]

The severe CD4, CD8 and NK cell lymphopenia that we and others described before, can now be better explained. Together with the up regulation of activation markers on PBMC in the severe group and lack of apoptosis, the observed lymphopenia likely results from recruitment of T cells to the site of infection, i.e the lungs with an parent lower cell count in peripheral blood. In this respect peripheral blood may not reflect the situation in all parts of the body.

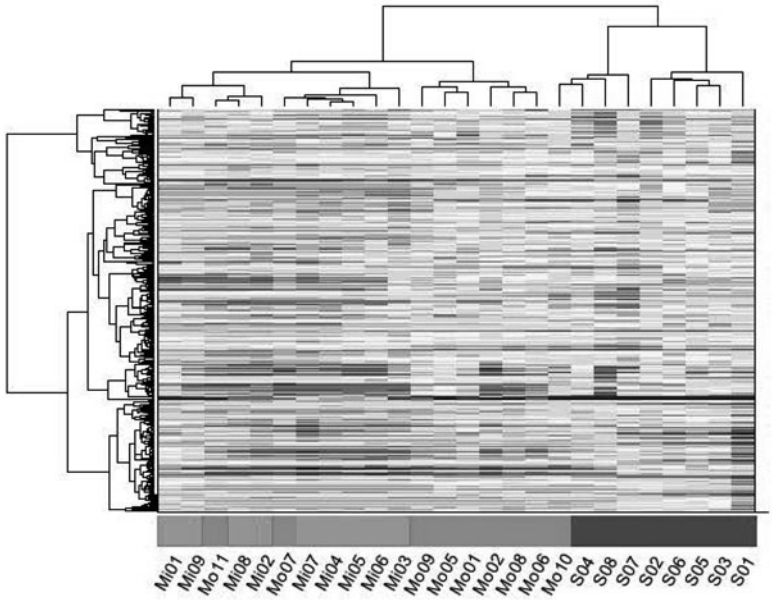
OLFM4 is associated with Th1 responses, an active or even overaggressive Th1 response may underlie the severe clinical manifestations in this group. This is reminiscent of interpretations in the early RSV vaccine trails. Nevertheless, more accurate measurements at the site of infection will be needed to determine whether in the severe cases an inadequate adaptive immune response or a hyper responsive reaction (for instance by excessive production of cytokines) is responsible for the severe manifestations of RSV infection.

Notwithstanding this uncertainty, in this study we are the first to show that OLFM4 transcription is associated with severity of disease in children with viral lower respiratory tract infections, also after correcting for age. These results emphasize the role of OLFM4 in innate and adaptive immunity and encourages further research into the presence of OLFM4 in PBMCs and the pathogenesis of RSV infections. Moreover, it could lead to the development of a new diagnostic tool to predict a severe course of viral respiratory disease and aid the physician in clinical decisions.

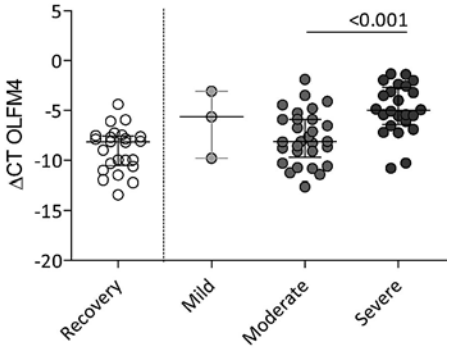
Supplemental



S1 Fig Transient lymphopenia during severe RSV infection. Immune phenotyping with flow-cytometry of circulating leukocytes of infants with severe RSV infection (n = 6) in the acute phase and after clearance of the infection (on average 4 weeks after discharge). The numbers of NK cells as well as CD4+ and CD8+ T cells return to normal, indicating that the lymphopenia was transient. Statistics were performed by paired students-t test, significance was set at $p<0.05$.



S2 Fig Discriminating mild disease from severe disease in children with RSV infection. 448 differentially expressed probesets were selected based on overlap in the comparison mild vs severe disease in RSV infected children and acute samples vs recovery samples of children with severe RSV infection. Samples were clustered based on these selected probesets by complete linkage hierarchical clustering with 1-correlation as a distance measure.



S3 Fig OLFM4 gene expression values in PBMCs from RSV+ infants during acute mild, moderate and severe viral lower RTI and after recovery. Expression levels are presented as ΔCt and median with inter quartile range (IQR). Statistics were performed by Kruskall Wallis tests ($p<0.001$), followed by Mann Whitney U tests for individual comparisons: mild vs moderate $p = 0.36$, moderate vs severe $p<0.001$, mild vs severe $p = 0.51$.

S1 Table Patient characteristics of validation cohort.

Values are given in numbers (percent- ages) and median and inter quartile range IQR

	Mild (N=14)	Moderate (N=42)	Severe (N=24)	*p*-value
Age (months)	3.2 [1.1-10.3]	4.9 [2.0-14.5]	1.2 [0.6-2.8]	$p<0.01$*
Gender (male)	8 (57%)	22 (52%)	12 (50%)	NS
Gestational age (wks)	36.8 [39.1-40.0]	38.0 [37.0-40.0]	38.9 [37.0-40.5]	NS
Length of stay (days)	3 [2-3]	6 [4-9]	11 [10-13]	$p<0.001$**
Confirmed RSV infection	3 (21%)	29 (69%)	15 (63%)	<0.01***

* mild versus severe $p = 0.05$, moderate versus severe $p<0.001$

** mild versus moderate and severe $p<0.001$, moderate versus severe $p<0.001$

*** mild versus moderate $p<0.01$, mild versus severe $p<0.05$

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10

Transcriptome assists prognosis of disease severity in respiratory syncytial virus infected infants

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Abstract

Respiratory syncytial virus (RSV) causes infections that range from common cold to severe lower respiratory tract infection requiring high-level medical care. Prediction of the course of disease in individual patients remains challenging at the first visit to the pediatric wards and RSV infections may rapidly progress to severe disease.

In this study we investigate whether there exists a genomic signature that can accurately predict the course of RSV. We used early blood microarray transcriptome profiles from 39 hospitalized infants that were followed until recovery and of which the level of disease severity was determined retrospectively. Applying support vector machine learning on age by sex standardized transcriptomic data, an 84 gene signature was identified that discriminated hospitalized infants with eventually less severe RSV infection from infants that suffered from most severe RSV disease.

This signature yielded an area under the receiver operating characteristic curve (AUC) of 0.966 using leave-one-out cross-validation on the experimental data and an AUC of 0.858 on an independent validation cohort consisting of 53 infants. A combination of the gene signature with age and sex yielded an AUC of 0.971. Thus, the presented signature may serve as the basis to develop a prognostic test to support clinical management of RSV patients.

Introduction

Respiratory syncytial virus (RSV) causes infections that range from common cold to severe lower respiratory tract infection that in some instances may have a fatal outcome. Especially infants, elderly and patients with underlying chronic disorders suffer from severe RSV infections[1,2]. In infants, RSV is the leading cause of lower respiratory tract infections (LRTI) and is responsible for 80% of acute bronchiolitis cases[3]. RSV infections pose a huge burden on society in terms of disease, logistics and socio-economic sequelae. There is an unmet need for an RSV vaccine, despite considerable research efforts no licensed vaccine has been developed. In industrialized countries, 1-5% of infants with RSV infection are hospitalized[4–7]. Some of these infants yet suffer from severe disease upon admittance, while others are admitted without severe symptoms since the course of bronchiolitis is highly variable and the need for supportive care cannot be predicted[8,9]. Several risk factors for developing severe RSV disease in infants have been identified, including preterm birth, young age, sex and environmental factors like in- house smoking[10]. Notwithstanding these known risk factors, current medical practice does not allow accurate prediction of whether an infant will further progress to severe RSV disease or not and could even be sent home safely. Genomic technologies have contributed to study the virus- host interaction, including virus discovery, pathogenesis studies, the design of antiviral strategies and identification of biomarkers to support clinical management of infectious diseases[11–14]. For RSV infections, this has supported the characterization of vaccine-induced skewed host responses upon infection[15,16]. Meijas et al.[17] recently used blood transcriptome profiles obtained within 3 days of hospitalization to characterize the host response to RSV infection in infants compared with rhinovirus or influenza infections and identified transcriptional profiles that associate with RSV disease severity. However, a prognostic model for RSV severity based on gene expression profiles collected at admittance to the hospital has not been developed. In this study we aim to identify and validate a gene signature that discriminates severe from less severe RSV LRTI that do not require advanced support. Such a signature together with other clinical parameters may improve the prognosis of less severe patients that could be safely sent home.

Material and Methods

Study design

Study subjects were recruited at Canisius Wilhelmina Hospital, Radboud University Medical Center, Nijmegen, and Erasmus Medical Center, Rotterdam, The Netherlands. Nasopharyngeal wash and blood samples were prospectively obtained from patients less than 2 years of age with a viral bronchiolitis. Patient enrolment occurred 7 days a week and samples were taken within 24 hours after first contact with the hospital. Seventy-three percent of all eligible bronchiolitis patients agreed to participate in the

study. The major reasons for non-inclusion were parental availability to sign consent and the hesitancy for the venipuncture. Only patients with an RSV infection, as retrospectively determined by PCR were included in the study. Exclusion criteria were: immunodeficiency, systemic steroid treatment in the previous 2 weeks, blood transfusion, congenital heart and chronic lung disease. A Tempus tube (Tempus™, Applied Biosystems, Austria) and sodium heparin tube were filled with 3ml of blood. Medical history, demographic and clinical data were collected from medical records and questionnaires. The (hospitalized) patients were followed until recovery and were retrospectively classified as: mild for children without hypoxia, moderate for patients requiring supplemental oxygen (oxygen saturations <90%, ≥10 minutes) and severe for children requiring mechanical ventilation due to apnea, exhaustion and/or respiratory failure. Recovery samples were obtained after 4-6 weeks, during home visits. Blood samples were obtained from healthy controls (HC) without underlying diseases or medication subjected to elective surgery.

Study approval

The study protocol was approved by the Regional Committees on Research involving Human Subjects of Arnhem-Nijmegen and Rotterdam and were conducted in accordance with the principles of the Declaration of Helsinki. Written informed consent was obtained from the parents of all children prior to inclusion in the study.

Sample processing and blood transcriptome profiling

Inclusion of patients and sample collection was performed by a single MD at the hospitals. Multiplex RT-PCR was performed to test the nasopharyngeal washes on 15 different viral pathogens, as previously described [18]. Blood was collected in Tempus tubes for immediate stabilization of RNA and subsequently stored at -80°C. Total RNA was isolated from each blood sample, processed, assessed, labelled and hybridized to a single Affymetrix Human Genome U133 plus 2 gene chips; and image analysis was performed in the same lab and by one technician as described in supplementary material. The raw data has been deposited in the ArrayExpress database under access number E-MTAB-5195.

Data preprocessing

Microarray data was preprocessed using R 3.1.2 [19] and Bioconductor [20]. Upon initial quality control and VSN normalization (to render samples comparable), probeset (a combination of multiple probes) summarization was performed by median polish [21,22]. Unless otherwise stated, all probesets/genes present on the Affymetrix GeneChip were used for data analysis. Samples were labelled and hybridized in two batches which did not correspond to any biological variable as samples were randomly assigned to the batches. The normalized expression values were adjusted for a batch effect (see supplementary Fig. S1) using *ComBat* [23]. Additionally, we assessed confounding effects of clinical parameters age and sex on gene expression–severity relationship using "biasograms" [24].

Differential expression analysis

To obtain a global view of the blood transcriptome changes in response to RSV infection (i.e. to evaluate whether whole transcriptome changes associate with severity), a principal component analysis (PCA) as an exploratory analysis was performed on the age by sex standardized data. Next, a differential expression (DE) analysis was performed on normalized-batch-adjusted data controlling for an age by sex effect using empirical Bayes linear models [25] implemented in the R package *limma* [26]. Details of the models are found in supplementary material. We controlled for multiple testing via false discovery rate (FDR) using a Benjamini and Hochberg procedure [27]. Gene set enrichment analysis was performed using Ingenuity pathway analysis (IPA, www.qiagen.com/ingenuity).

Identification and evaluation of prognostic biomarkers

Since we are interested in identifying RSV-infected infants that will progress to severe stage upon presentation to the hospital, we grouped mild and moderate samples and aimed to separate these samples from infants that were presented with or progressed to severe disease after hospitalization. We chose to utilized probabilistic predictors (to predict the chance of an RSV- infected infant to be severe) because in clinical applications, probabilities are more informative than absolute yes or no predictions [28]. Several probabilistic predictors exist in the literature and their performance depends on the type of the data they are being applied on [29]. Using results of [29,30] and observed correlations in the data, three probabilistic classification functions that could be optimal for this data were chosen as described in supplementary material. These functions were support vector machines (SVM) [31], shrunken centroids discriminant analysis (SCDA)[32] and random forest (RF) [33].

For each classification function, the experimental data was split into a learning set and a test set using leave-one-out cross-validation (LOOCV). Cross-validation reduces optimistic bias by ensuring that our models are evaluated on an independent dataset that was not used to constructed these models. Most probabilistic classification functions require hyper-parameters to perform variable selection among the huge number of variables (probesets). Usually, the best values for these hyper-parameters are also determined by cross-validation. Thus, the parameter(s) of the function were optimized using an inner loop of five-fold cross-validation on the learning set. Next, a prognostic model was built with the optimal parameter(s) on the entire learning set and evaluated with the test set, as described in supplementary material. The following R packages; *CMA* [34], *e1071* [35], *pamr* [36] and *randomForest* [37] were utilized for class prediction. The best calibrated and refined function amongst the three functions was selected and its performance evaluated using the area under the receiver operating characteristic (ROC) curve (AUC). Finally, the transcripts that maximized the binomial log-likelihood function, with the leave-one-out cross-validated data were retained as a gene signature from the selected function as described in supplementary material.

Comparison of biomarkers to clinical parameters

Age and sex are readily available clinical parameters that have been determined to be associated to RSV disease severity [38]. To assess the gain attained with a genomic model over a model with these clinical parameters, and the effect of standardization, the leave-one-out cross-validated predicted probabilities of progressing to severe for all samples were transformed to genomic scores (a genomic score is single measure of the genome of a sample as predicted by a model) for models with unstandardized and standardized data. Logistic regression models (see supplementary material) were then fitted with the genomic scores and/or clinical parameters and their AUCs compared.

Validation of biomarkers

For an independent validation, a subset of the Illumina RSV data of Meijas et al. [17] was used. Since the experimental data and validation data were obtained using different platforms, we linked the data using gene symbols and applied cross-platform transformation (to render gene expression comparable across datasets) as described in supplementary material. The transformed data was supplied to our prognostic model for predictions of probabilities of severity. For a confirmatory analysis of how well our prognostic model performs, we built and evaluated a prediction model with the chosen function (same function used to build our prognostic model) on the entire Illumina data and compared our validation performance to the performance from this (unrestricted) data.

Results

Study subjects and sampling

Thirty-nine infants hospitalized with acute RSV bronchiolitis were included in the study. Nasopharyngeal wash and whole blood samples for mRNA profiling were collected within 24 hours upon hospital admittance. **Table 10.1** presents the characteristics of the study subjects. As expected, patients with the most severe course of RSV bronchiolitis were significantly younger than those with a relative mild or moderate course of this disease. The variables related to disease severity; duration of oxygen, and length of stay in the hospital were highest in the severe group, with ventilation indicating the method by which oxygen was supplied. The proportion of co-infections was lower in severe patients as compared to the other severity categories. There were no differences in the occurrence of other known risk factors.

Age and sex as confounders of gene expression–severity relationship

Figure 10.1a and b respectively illustrate the confounding effects of sex and age on the gene expression-disease severity relationship. These figures show that whereas age is negatively correlated to severity, sex is uncorrelated to severity. Nevertheless, the high positive/negative correlations of a considerable number of transcripts to sex and age, as well as severity, indicate a confounding effect of these variables on the expression-

Table 10.1 Patient characteristics (n represents the number of samples per group)

Parameters	Mild (n=7)	Moderate (n=14)	Severe (n=18)
Age (days)	153 [84, 291]	185 [60, 333]	31 [17, 76]
Gestational age (weeks)	40 [29, 41]	40 [37, 41]	39 [37, 40]
Birth weight (kg)	3.5 [3.0, 4.2]	3.4 [3.1, 3.9]	3.3 [2.5, 4.0]
Symptomatic days	4 [2, 6]	4 [3, 6]	3 [2, 4]
Duration on O2 (days) Ventilation	0 None	3 [2, 5] Supplemental	8 [7, 11] Mechanical
Length of stay (days)	4 [2, 6]	5 [3, 8]	11 [9, 13]
Breastfeeding	4 (57)	11 (79)	12 (67)
Male gender	5 (71)	10 (71)	12 (67)
RSV + other virus(es)	4 (57)	8 (57)	3 (17)

Data are presented as median and interquartile range (IQR) in square brackets [,] or number and percentage in brackets ().The median age of the healthy controls was 536 days (IQR [472, 602]).

severity relationship of these transcripts, thus warrant adjustment. **Figure 10.1d and e** illustrate the "biasograms" after an age by sex standardization. These figures show that standardization has no effect on severity correlated transcripts but as expected, transcripts that were originally correlated to age and sex become uncorrelated. A positive correlation of age to sex which signifies an age by sex interaction as a potential confounder on the gene expression-severity relationship was also observed (**Figure 10.1c**) and eliminated after standardization (**Figure 10.1f**).

Global blood transcriptome profiles associate with RSV disease severity

Figure 10.2 illustrate a PCA on the whole transcriptome and the first principal component accounts for 25% of the variance in the transcriptomes and associates with disease severity. Transcriptome profiles of HC and recovery samples group together on the first principal component and are located opposite to profiles of severe infants. The distinct groups do not form discrete clusters in the PCA but gradually shift from mild through moderate to severe, with considerable overlap. This shows that the blood mRNA profiles substantially capture the severity of lower respiratory tract RSV infection.

Number of differential gene expression relates to RSV disease severity

Table 10.2 presents results of differential gene expression analysis and reveals that the number of DE transcripts increases with disease severity. No DE transcript was identified between mild versus HC samples when applying a FDR of 5% and absolute fold change (FC) threshold of 2. However, 17 and 221 transcripts were DE between moderate and severe versus HC respectively. Interestingly, all transcripts that are DE in moderate class are also DE in severe class with larger FC. About 90% of these DE transcripts are up-regulated. Comparison of HC with recovery samples revealed a

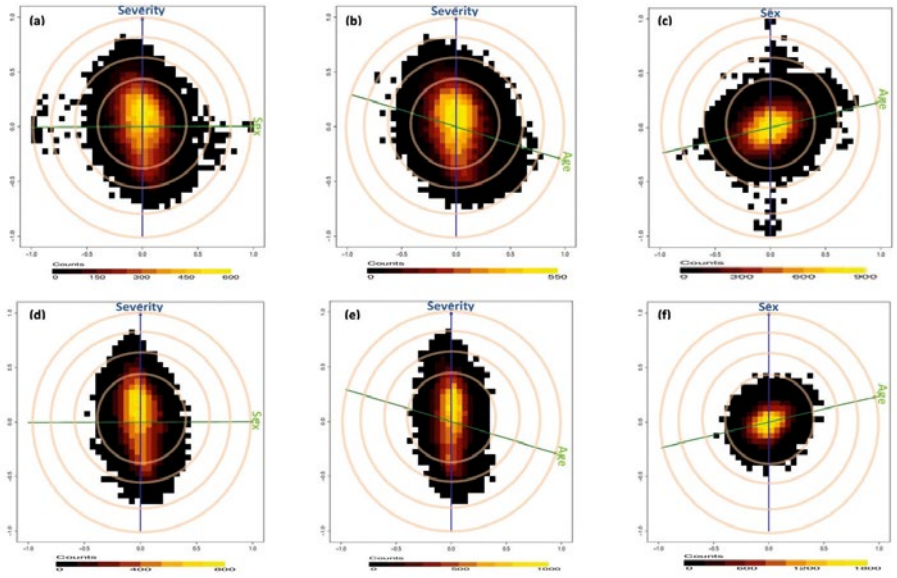

Figure 10.1 Confounding effect of Sex, Age and Age by Sex on gene expression–severity relationship, before: (**a**), (**b**) & (**c**) and after: (**d**), (**e**) & (**f**), an age by sex standardization. The blue and green lines represent the clinical variables, the cosine of the angle between the lines represents its correlation to the blue line (Sex is not correlated to Severity, Age is negatively correlated to Severity i.e. younger kids become severe and Age is positively correlated to sex i.e. females are older). The cloud of points represent the transcripts and their correlations to both variables with most transcripts uncorrelated to the variables (yellow cloud) while a considerable number (black cloud) are correlated to Severity, Sex, Age and Age*Sex. The associations between the transcripts and Sex, Age or Age*Sex are significantly eliminated after standardization while retaining that of Severity.

single down-regulated transcript while moderate versus mild yielded no DE transcript, severe versus mild or moderate yielded 178 and 49 DE transcripts respectively. Lastly, 95 transcripts were DE between severe versus combined mild/moderate samples.

RSV induced blood transcriptome profiles reveal an inflammatory response

Figure 10.3 shows that multiple relevant categories of molecular and cellular functions are significantly enriched when comparing severe to HC samples. With “Cell-to-Cell Signaling and Interaction” top category, gene sets related to activation of several types of immune cells including lymphocytes, granulocytes and specifically neutrophils are most significantly enriched. In addition, gene sets that are involved in migration and tissue infiltration of these same activated cell types are most significantly enriched within the category “Cellular Movement” that ranks third on this figure. Finally, several

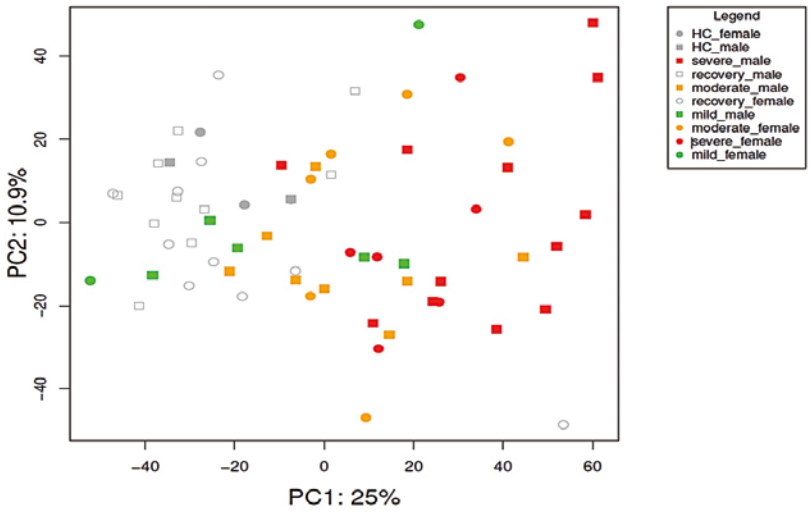

Figure 10.2 Global blood transcriptome profiling with principal component analysis: the first principal component (PC1) accounts for 25% of the variance in the dataset and associates with disease severity. This can be observed as a shift from healthy controls and recovery cases (left) through mild and moderate to severe cases (right), with considerable overlap.

Table 10.2 Number of differentially expressed transcripts for each contrast at FDR of 5% and absolute fold change cutoff of 2.

	Mil-HC	Mod-HC	Sev-HC	Mod-Mil	Sev-Mil	Sev-Mod	RC-HC	Sev-(Mil+Mod)/2
UP	0	15	194	0	164	42	0	82
Down	0	2	27	0	14	7	1	13
Total	0	17	221	0	178	49	1	95

Where Mil: Mild, Mod: Moderate, Sev: Severe, HC: Healthy controls (<2years) and RC: Recovery samples.

high ranking molecular and cellular function categories and their underlying gene sets indicate the immune cells involved are strongly proliferating. A list of genes involved in each of these pathways is presented in supplementary **Table S1**. Taken together, blood transcriptome changes in RSV disease reveal a typical inflammatory response to a viral infection.

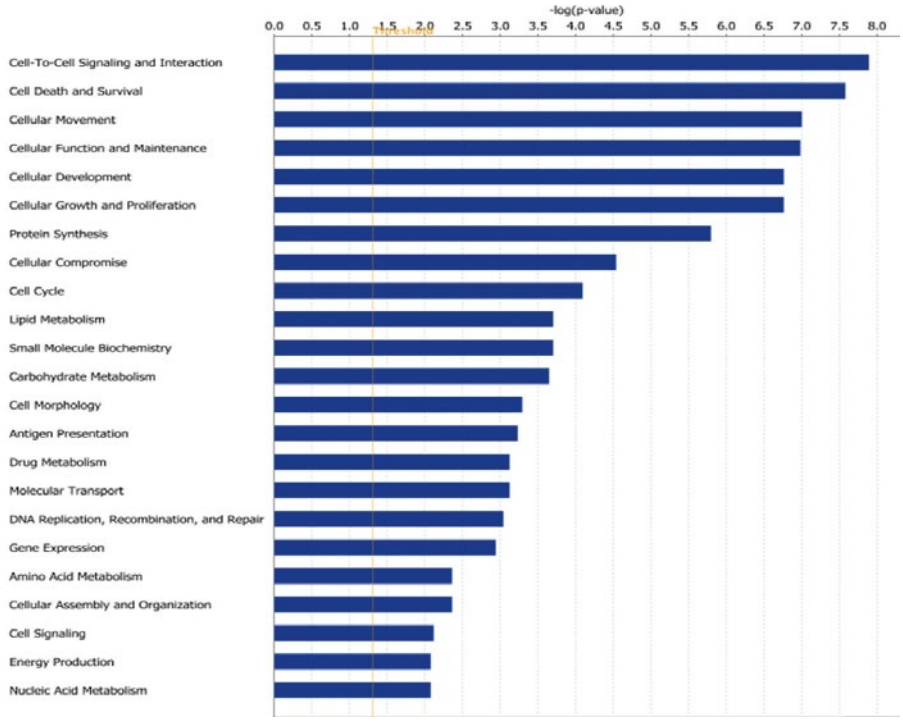

Figure 10.3 Ingenuity pathway analysis (IPA) Molecular and Cellular functions gene set analysis for severe vs healthy control contrast.

Early blood transcriptome changes to predict a severe outcome of RSV infection

To construct a predictive model, we combined mild and moderate cases as a single group and three probabilistic classification functions were chosen based on supplementary **Figure S2** and results of Jong et al. and Kim & Simon. [29,30]. Using these functions, classifiers were built and evaluated using LOOCV on the experimental data. SVM was chosen as the best calibrated and refined as shown on supplementary **Figure S3** and henceforth considered for all analyses. The LOOCV predicted probabilities from SVM were used to evaluate its performance and are plotted on **Figure 10.4a** against the true RSV status as retrospectively determined. This figure shows that 5 samples out of 39 were misclassified at a 50% cutoff and when applying a proposed uncertainty band of 30-70% just one false negative is witnessed. Evaluation of the clinical characteristics of the single false negative patient as well as those patients with uncertain predictions (plotted within the proposed uncertainty band) did not reveal any recognizable pattern. The false negative patient had uniquely RSV and only a single patient plotted within the

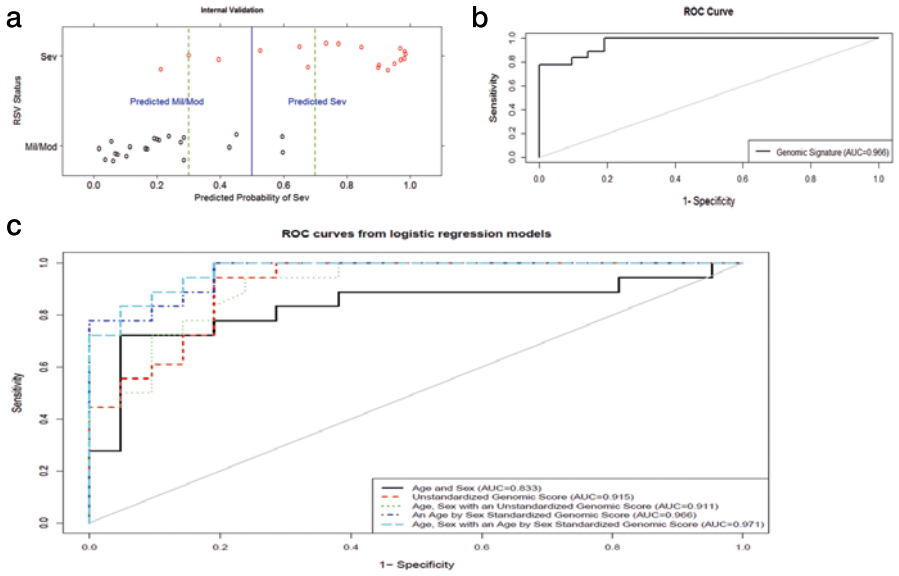

Figure 10.4 Internal validation of gene signature: (**a**) samples' predicted probabilities of being severe. (**b**) shows the ROC curve and the AUC for predicted probabilities. The AUC value of approximately 1 indicates how accurate our signature performs on this internal validation set. (**c**) shows that a genomic model from the age by sex standardized data out performs that from the unstandardized data. In addition, there is a significant difference between a model with clinical parameters and that with a genomic score and just a slight improvement when both parameters are included in a model.

uncertainty band had RSV + other virus(es). **Figure 10.4b** presents the corresponding ROC curve from the LOOCV predicted probabilities and AUC of 0.966 demonstrates the high discriminative power of our prognostic model.

Genomic biomarkers outperform clinical parameters age and sex

Figure 10.4c presents the ROC curves from logistic models of clinical parameters and/or genomic scores. The genomic score model from the standardized data (AUC=0.966) outperforms that from the unstandardized data (AUC=0.915). In addition, there is a significant difference between a model with age and sex only (AUC=0.833) and that with a standardized (AUC=0.966) or unstandardized genomic score (AUC=0.915). Whereas there is a slight improvement from clinical parameters and standardized genomic score model (AUC=0.971), there is no improvement from the clinical parameters and unstandardized genomic score (AUC=0.911), indicating that indeed standardization completely removed an age-by-sex effect on the gene expression data.

An 84 gene expression signature predicts absence of disease progression

To extract the prognostic signature, we selected top transcripts maximizing the binomial log- likelihood function using LOOCV predicted probabilities as illustrated on supplementary **Figure S4**. This figure depicts a 1-SE maximum of 95 transcripts corresponding to 84 unique genes, which are displayed on **Table 10.3**. Of the 95 transcripts constituting the prognostic signature, 81 (85.26%) were found to be significantly DE between severe and non-severe patients (FDR cutoff of 5%, supplementary **Table S2**). The inclusion of non-DE transcripts in the classification model is expected, since not only DE genes are instrumental in class discrimination as illustrated by the two-dimensional scenario in supplementary **Figure S5**.

Performance of the genomic signature retained on an independent dataset

For an independent validation, a subset of the Illumina RSV data of Meijas et al. [17] was used. Since the experimental data and validation data were obtained using different platforms, we linked the data using gene symbols and applied cross-platform transformation (to render gene expression comparable across datasets) as shown on supplementary **Figure S6** and extensive described in the supplementary material. **Figure 10.5a** presents

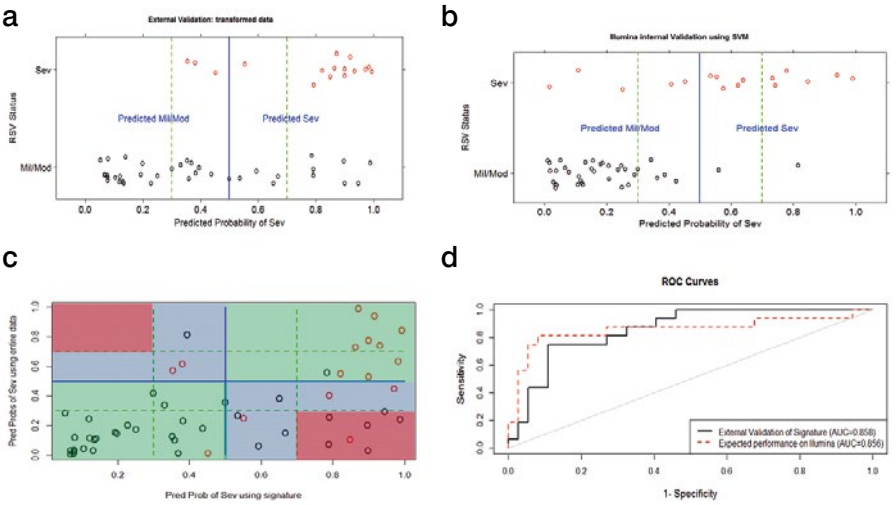

Figure 10.5 Predicted probabilities of being severe from the validation data against true RSV status using; our diagnostic signature (**a**) and LOOCV on unrestricted data (**b**). (**c**) illustrates the agreement of the predictions from both models, green regions are perfect agreement, blue are disagreements at a 50% cutoff and red are disagreements at a 30% -70% uncertainty band. Finally, (**d**) presents the ROC curves and AUC from both models illustrating similar AUC values.

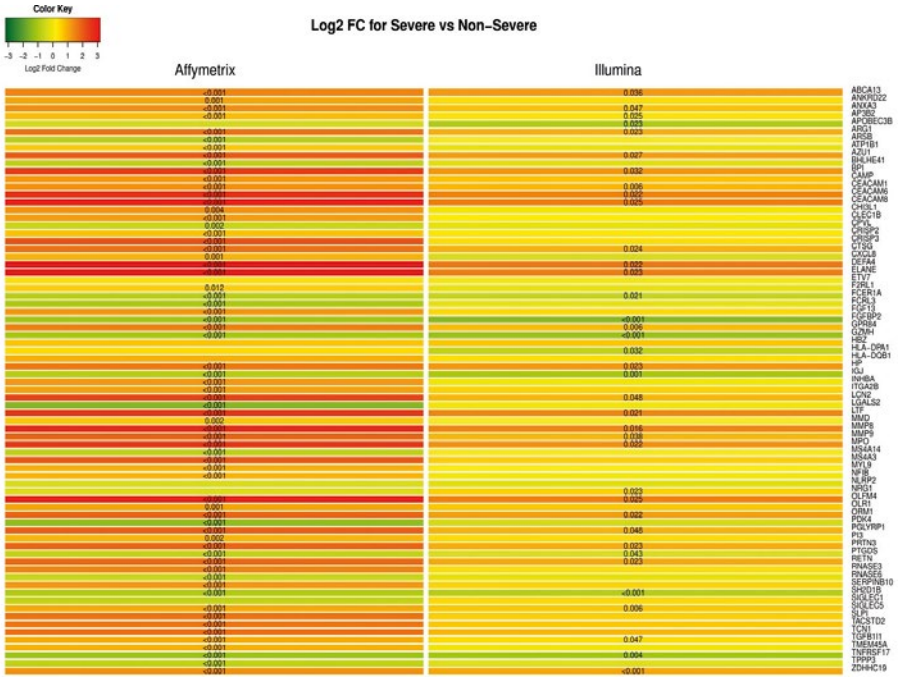

Figure 10.6 Log2 Fold change between Severe vs Non-Severe infants for 75 common genes in Affymetrix and Illumina datasets. Red represent up-regulation while green represents a down-regulation and the significant FDR adjusted p-values are placed in the cells. As one can clearly see, there is a huge overlap in the direction expressions across datasets. Where there are slight differences, these differences are not significant as shown by a non-significant p-value in at least one of the datasets.

predicted probabilities of severe on the validation data using 75 of our 84 prognostic gene signature that were common in both experimental and validation datasets, while **Figure 10.5b** presents the LOOCV predicted probabilities from SVM on the entire Illumina data. Both figures show that using the unrestricted data leads to more certain probabilities and slightly improves specificity compared to our signature. Nonetheless, **Figure 10.5c** illustrates a large agreement between the predicted probabilities of the two models, while Figure 5d clearly reveals that both models are alike as demonstrated by the AUCs of 0.858 and 0.856 for our signature and the unrestricted model respectively. To assess the concordance of the expression patterns of our signature on both datasets (Affymetrix and Illumina), we plotted the log2 fold changes of the common 75 genes as shown on **Figure 10.6**. From this figure, one clearly sees that there is a huge concordance in the direction of expressions across datasets. Where there are slight

differences, these differences are not significant as shown by a non-significant p-value in at least one of the datasets.

Discussion

RSV infection in infants may cause life-threatening disease. No vaccine is yet available and triage of patients is challenging since RSV infections may rapidly progress to severe disease. No reliable prognostic model to predict which RSV patient will not progress to severe disease and could be safely send home is available either. Thus, clinical care is symptom-based and a significant proportion of RSV infected infants is hospitalized for observation purposes. We have provided an 84 gene signature that discriminates hospitalized infants with less severe RSV infection from those infants with severe RSV disease. The identified signature yielded a LOOCV AUC of 0.966 on the experimental data and was independently validated with an AUC of 0.858 and might serve as a basis to develop a prognostic test for clinical management of RSV disease.

In line with epidemiological observations [38] and observations of Mejias et al [17], we showed the confounding effects of age and sex on gene expression-severity relationship for RSV disease. Studies in any RSV patient cohort with a naturally occurring "skewed" distribution of age and sex can be standardized for these parameters. By adjusting for an age-by-sex effect in our analyses, we obtained age-by-sex independent results which can be effectively applied to any patient(s). The high performance of our signature on the age and sex matched validation data signifies age-by-sex independence and robustness of this signature. Fewer co-infections were observed in severe patients (**Table 10.1**). A similar trend has been described previously [39]. In our cohort study we did not take into account co-infections since no consistent association between the occurrence or absence of co-infections with RSV disease severity have been reported [39–43]. Furthermore, we aimed at the identification of a gene signature in a natural "real-life" cohort of patients not stratified according to age or occurrence of co-infections. We hypothesized that changes in blood cell type distribution and/or mRNA expression changes of the circulating cells collected from peripheral blood reflect local lung host response characteristics that associate with disease severity. PCA and DE analysis indeed revealed significant changes in the transcriptome profile of whole blood. Gene set analysis further shows that relevant processes are monitored including the activation, migration and tissue infiltration of lymphocytes, granulocytes and neutrophils. Individual DE genes in severe RSV disease revealed overexpression of the neutrophil associated genes MMP8 and MMP9, which have previously been related to severe RSV disease [44]. ARG1 and CHI3L1 that have been linked to alternatively activated macrophages in a mouse model for vaccine enhanced RSV disease [16] were also found to be strongly up-regulated. This suggests that the collected blood transcriptome profiles indeed reflect local lung host response.

In our class prediction analysis, three functions were evaluated and the best was chosen. While it has been pointed by [45–48] that selecting a minimal-error classifier leads to selection bias that should be corrected, the literature does not stipulate a selection bias when using calibration and refinement scores as evaluation measures. Nevertheless, we employed the nested cross-validation correction of selection bias [46] in our model building procedure by splitting our experimental data into learning and test sets with an inner loop split on the learning set for parameter(s) optimization. Though found to contain high variance, we utilized leave-one-out cross-validation for the test set because it yields approximately an unbiased estimate of the true (expected) prediction error [49] and because we were interested in the individual sample predicted probability of severe and not entirely on the expected predicted error. Nevertheless, where we were interested in the expected predicted error, as in the optimization of parameters, we utilized five- fold cross-validation as recommended by Breiman and Spector [50]. To validate the identified signature, an independent dataset generated on a different platform was used. Despite (i) the several sources of variability between our experimental data and the validation data that stem from - but not limited to - array platforms and different clinical cutoffs of RSV severity statuses, (ii) different time of profiling, 1-3days after hospitalization and (iii) loss of information due to a reduction in signature because of no corresponding transcripts on Illumina platform and the aggregation of multiple transcripts to genes, our signature yielded an AUC of 0.858 that was comparable to accuracy (AUC of 0.856) when using the Illumina data (validation set) as experimental set. Cross-platform validation is rare due to lack of guidance on how this can be done reliably. We presented a cross-platform validation procedure.

The RSV patients enrolled in the study displayed varying disease severities but were all hospitalized thus representing a severe disease enriched subset of RSV infected infants. The patients enrolled however also represent a natural cohort of patients including a significant number of patients that eventually did not require extensive medical care and could have been discharged home. Since the blood samples were collected soon after hospital admission, the generated blood transcriptomes and the derived gene signature may serve as a basis for the development of a novel genomic tool to support clinical management of RSV disease including triage of patients presenting at the hospital provided that a rapid (real time) gene test can be developed. Larger transcriptome data sets are however required to construct predictive models that may also allow for discriminating mild from moderate and moderate from severe cases. Ultimately, one would like to extend the RSV biomarker program to earlier time point samples (e.g. obtained when visiting a general practitioner) and to samples collected from patients infected by other (respiratory) infectious agents or pathological conditions (comorbidities) in order to identify specific respiratory viral prognostic biomarkers. To this end a novel gene signature have to be developed using a much larger early blood sample cohort. The current results support the development of diagnostic tests for personalized medicine that not only provide information on the causative infectious agent, but also about the disease severity that may be expected.

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11

Gene expression analysis reveals an important role for neutrophils in severe viral respiratory tract infections in children

Submitted

Abstract

Objectives: The pathophysiology of severe respiratory syncytial virus infections in infants remains to be elucidated. Gene expression analysis of peripheral blood was used to identify differences in the immune response of infants with severe RSV infection.
Methods: Affymetrix microarray analysis was performed on whole blood of 51 patients <2 years of age with viral lower respiratory tract infection and on 12 age-matched healthy controls. Transcriptional modular framework analysis was applied and gene expression-based distance to health was calculated. Subsequently, micro-arrays on neutrophils and PBMC were performed and blood smears were analyzed.
Results: Gene expression-based distance to health correlated significantly with disease severity, duration of supplemental oxygen and length of hospitalization. The neutrophil associated module was most discriminative for patients with a severe RSV infection. This signature was partly caused by cell shift as shown by cell counts and neutrophil specific micro-arrays. In addition, neutrophil specific signatures in the PBMC enriched fraction of peripheral blood from severely ill patients was found.
Conclusion: Neutrophil transcriptome signatures correlate with severe RSV disease. Low density granulocytes co-purified during density gradient PBMC isolation are only present during acute RSV infection in children and may have an unexpected role during severe respiratory viral infections in children.

Introduction

In 2004 the WHO estimated that 78% of all worldwide mortality for children under the age of five years was caused by only six diseases. Acute respiratory tract infections (ARIs) and diarrheal diseases are number one in this ranking, each responsible for 17% of deaths. Millions of children are affected by ARIs each year accounting for nearly 2 million deaths per year.[1] *Respiratory Syncytical virus* (RSV) is the third most common pathogen in cases of childhood death due to ARI, after S*treptococcus pneumoniae* and *Haemophilus Influenzae* type b.[2] Whilst treatment and protective vaccines are available for the bacterial causes of ARI, no vaccine or treatment exists for the majority of viral respiratory pathogens, including RSV. Disease severity of respiratory viral infections can range from a common cold to severe bronchiolitis with respiratory insufficiency, resulting in the need for mechanical ventilation. Especially infants, the elderly and patients with underlying chronic disorders are vulnerable for a severe course of viral respiratory infections. Although risk factors for severe disease in infants are known, the majority of infants on the ICU have no underlying co-morbidities. A combination of physical factors, the developing immune system, genetic background and immune modulatory capacities of the virus are thought to be important. [3] Nonetheless, although all infants have an immature immune system, only 1-10% of RSV infected infants need hospitalization.[4,5] Hence, it is important to further elucidate the mechanisms behind the development of severe RSV disease. The use of microarray studies in adults and children has proven to be useful in gaining insights in the immune responses against pathogens. Studies comparing the gene expression profiles of patients suffering from different diseases have shown that the host response is very specific, enabling the identification of different etiologic viruses or bacteria.[6-10] In addition, this host response gives information about underlying pathophysiology and mechanisms involved in the immune response against the pathogens.[6-8,11] It has been shown that disease severity can be determined based on the host response.[7,12] Chaussabel *et al.* described an alternative method to analyze gene expression data, based on modules of co-expression of genes.[13] This results in lower error rates and more comprehensive results, providing a better insight in the pathophysiology of disease.[13] Studies using this module showed a distinctive response of infants to RSV infections compared to other viruses.[7] In this study, we further explore the immune response of infants with severe respiratory syncytial viral infections. By comparing gene expression from patients with different disease severities we intend to improve the understanding of the pathophysiology of severe disease.

Methods

Study design

Nasopharyngeal wash and blood samples were prospectively obtained from patients < 2 years of age with a viral bronchiolitis. Subjects were recruited at Canisius Wilhelmina Hospital and Radboud university medical center, Nijmegen, the Netherlands. Patient enrolment occurred 7 days a week and samples were taken within 24 hours after first contact with the hospital. The inclusion rate was 73%. The major reasons for exclusion or refusal were parental availability to sign consent and the hesitancy for the venipuncture. Exclusion criteria were: immunodeficiency, systemic steroid treatment in the previous 2 weeks, blood transfusion, congenital heart and chronic lung disease. A Tempus tube (Tempus™, Applied Biosystems, Austria) and sodium heparin tube were filled with 3 ml of blood. Medical history, demographic and clinical data were collected from medical records and questionnaires. Patients were followed until recovery. At that point the patients were classified into three severity groups: mild for children without hypoxia, a moderate group for patients requiring supplemental oxygen (oxygen saturations <93%, ≥10 minutes) and a severe group with children requiring mechanical ventilation. Recovery samples were obtained after 4-6 weeks, often during home visits.

A healthy control group for the microarray was obtained from patients without underlying diseases or medication, who needed a venipuncture for screening purposes. A total cohort of 51 acute, 31 recovery and 12 healthy control samples were available for analysis. Only patients with an RSV infection were included in this study.

Material processing

Multiplex RT-PCR was used to test the nasopharyngeal washes on 15 different viral pathogens, as previously described [14]. From the blood in the sodium heparin tubes a thin peripheral blood smear stained with Giemsa was made to determine the percentages of PBMC and granulocytes. The remainder was diluted 1:1 and added to 5 ml lymphoprep (Lymphoprep®, Axis Shield, Norway) in a 15 ml Falcon tube for density gradient centrifugation. Plasma was obtained and PBMC and granulocytes were harvested. The neutrophils were subsequently lysed in isotonic ice-cold NH_4Cl solution (8.3 g/L NH_4Cl, 1 g/L $KHCO_3$ and 37 mg/L EDTA) and washed. Both cell subsets were stored in Trizol® reagent (Invitrogen, The Netherlands) at -80°C. The Tempus tube was directly stored at -80°C.

RNA isolation and microarray gene expression analyses

RNA from Tempus tubes was isolated following the manufacturers protocol. RNA from granulocytes and PBMC was isolated with Trizol following the manufacturers protocol and subsequently cleaned with Qiagen RNA Clean up kit. RNA integrity and quality was assessed using capillary electrophoresis [RNA 6000 Nano LabChip (Agilent)] on an Agilent Bioanalyzer 2100 system. RNA processing, target labelling and hybridization to gene expression arrays was performed by standard methods as described [15]. Biotin

labeled cRNA was obtained using the One-Cycle Eukaryotic Target Labelling Assay (Affymetrix). 15 μg of fragmented, biotin labelled cRNA was hybridized to Affymetrix® GeneChip® Human Genome U133 plus 2.0 arrays according to standard Affymetrix protocol (Affymetrix Inc, Santa Clara, CA).
Quality control analyses were performed as previously described. [15,16] Scanned images were inspected for artifacts, percentage of calls present (<25%) and controls of RNA degradation. Upon initial quality control and VSN normalization, probeset summarization was performed by median polish. [17,18] Samples were labelled and hybridized in two batches. The normalized expression values were adjusted for a batch effect using *ComBat*. [19] Differentially expressed genes were identified based on log2 fold changes and false discovery rates (FDR). FDR adjusted *p*-values were calculated using LIMMA. Corrections for false discovery rates (FDR) were performed via the Benjamini Hochberg method [20] Microarray data have been deposited in ArrayExpress database under access number xxx. All microarray experiments were performed according to the MIAME guidelines.

Modular frameworks and distance to health

First a normal range of gene expressions per gene was defined based on whole blood gene expression data from healthy controls. This resulted in a bandwidth of health. Subsequently, gene expressions of every individual patient and healthy control were compared to this bandwidth of health. Genes with a significant p-value ($p<0.05$) and at least 1 log2 fold difference were used for subsequent calculations. The fold changes were transformed into absolute numbers and summed to create a distance to health score. Furthermore, genes where assigned to modules as defined by Chaussabel et al. Translations from the Illumina probe assigned modules to Affymetrix probes was performed using DAVID [21] based on gene symbols. Fold changes of significantly changed genes were summed per module and divided by the total amount of genes in that specific module to correct for the module size. Clustering of samples was performed on the module data using Euclidian distance correlations. Visualization was performed using iTOL. [22]

Cytometric bead array

The concentration of IL-6, G-CSF, IP-10, IL-8, IFN-γ, IL-1β, IL-10, TNF, MCP-1 and RANTES (category number 558276, 558326, 558280, 558277, 560111, 558279, 558274, 560112, 558587, 558324, respectively) in two fold diluted plasma was measured with Cytometric Bead Array (CBA BD™ Flex sets, BD biosciences) according to the protocol provided by the manufacturer. Cytokine concentrations could be measured from zero up to 10000 pg/ml.

Microarray data from neutrophil subsets

Affymerix CEL-files from a published study by de Kleijn et al. was downloaded from the Gene Expression Omnibus using their study coded GSE42358.[23] In this study

micro-arrays were performed on three subsets of neutrophils based on CD16/CD61L expression, derived from healthy volunteers four hours after LPS infusion. A list from genes that were at least one fold differentially expressed between the young versus the normal and activated neutrophils. This list was used to cluster the neutrophil micro-arrays from the RSV infected infants.

Statistics

The distribution of categorical variables is presented as percentages. Numerical variables are presented as mean with standard deviation (SD) or medians with inter quartile range (IQR) depending on the distribution of the data (Kolmogorov-Smirnov's test, $p>0.05$). Kruskal-Wallis test was used to compare 3 or more groups and Mann-Whiney U for comparisons between two groups. The Kendall's Tau test was used to study trends in non-parametrically distributed data. Corrections for false discovery rates (FDR) were performed via the Benjamini Hochberg method. [20]

Study approval

The study protocols were approved by the Regional Committee on Research involving Human Subjects Arnhem-Nijmegen (serving as the IRB) and were conducted in accordance with the principles of the Declaration of Helsinki. Written informed consent was obtained from the parents of all children.

Results

Of the 51 included infants, 40 had an acute lower respiratory tract infections due to RSV infections and 12 healthy controls were included in this study. Recovery samples were available for 20 patients. Patients with a severe course of disease were significantly younger than those with a mild or moderate course of disease(Kendall's tau correlation coefficient for trend -0.41, p=0.001, **Table 1**). There were no differences in the presence of other known risk factors. As expected the variables related to disease severity, duration of supplemental oxygen and duration of supplemental oxygen were highest in the most severe group (Kendall's tau correlation coefficient 0.80 and 0.59, $p<0.0001$). The median age of the healthy controls was 300 days [180-562 days] and half of them were male.

Distance to health correlates with different severity measures and enables clustering on disease severity. Calculation of a distance to health (DTH) per patient was performed by summing the fold changes of all significantly changed genes compared to the healthy controls. Euclidian distance correlations where determined of the samples based on the distances to health, the majority of infants clustered with other patients that experienced the same severity of disease (**Supplemental figure 1 and Figure 1A)**, while the majority of recovery samples clustered with the healthy controls **(Figure 1A)**.

Table 11.1 Patient characteristics.

	Mild (n=7)	Moderate (n=15)	Severe (n=18)	p-value
Age (days)	153 [84-291]	185 [60-333]	31 [17-76]	<0.01*
Male gender	5 (71)	10 (67)	12 (67)	NS
Gestational age (wk)	40 [39-41]	40 [37-41]	39 [37-40]	NS
Birth weight (kg)	3.5 [3.0 – 4.2]	3.4 [3.1-3.9]	3.3 [2.5-4.0]	NS
Breastfeeding	4 (57)	11 (73)	12 (67)	NS
Symptomatic (days)	4 [2-6]	4 [3-6]	3 [2- 4]	NS
Duration O2 (days)	0	3 [2-5]	8 [7- 11]	<0.0001**
Length of stay (days)	4 [2-6]	5 [3-8]	11 [9-13]	<0.0001***

Data are presented as median and interquartile range (IQR) or number with percentage, a Kruskal-Wallis test was performed for groups and a Mann-Whiney U test for individual comparisons. * mild vs moderate and severe p< 0.001, trend test correlation coefficient - 0.412 p<0.01. ** All comparisons p<0.0001, test for trend correlation efficient 0.807, p<0.0001. *** All comparisons p<0.0001, test for trend correlation efficient 0.585, p<0.0001.

The distance to health score increased from healthy towards a severe course of disease (Kendall's tau for trend 0.461, p<0.0001) **(Figure 1B)**. This measure also correlated with age, duration of supplemental oxygen and duration of hospitalization, Kendall's tau of -0.296, 0.322, 0.314, all p<0.001 **(Figure 1C and D)**. To assess whether patients fully recovered after 4-6 weeks, the DTH was calculated from the recovery group as well. Not all patients were fully recovered after this period in time based on the DTH (median 4905.2, 5111.5, 5787.7, 6068.1 and 7666.5 for healthy, recovery, mild, moderate and severe samples, respectively).

Six modules are differentially expressed among the different disease severities. To determine which modules were responsible for an increased DTH and thus severity of disease, we compared the modular scores across the severity groups. Out of 253 modules, 73 were differently expressed among healthy controls and RSV infected infants, after correcting for multiple testing 40 modules remained significantly changed (p<0.05). In addition, 47 modules were significantly changed among the three severity groups in acute disease. Six modules remained significant after correction for multiple testing, of which the most differentially expressed module is the neutrophil module (M5.15). In this module upregulation correlates with increasing disease severity (**Figure 2A**). The other significant modules were the cytotoxic T cell / NK cell modules (M3.6 and M8.46) which show increased downregulation (**Figure 2B,C**), while the inflammation (M4.2) (**Figure 2D**), platelets (M1.1) (**Figure 2E**) and erythrocyte module (M2.3) (**Figure 2F**) are all upregulated with increased disease severity. When the 253 modules are combined in 22 groups with distinct cellular functions [24], the same significant differences were observed within the following groups: neutrophils, Cytotoxic/NK cells, inflammation,

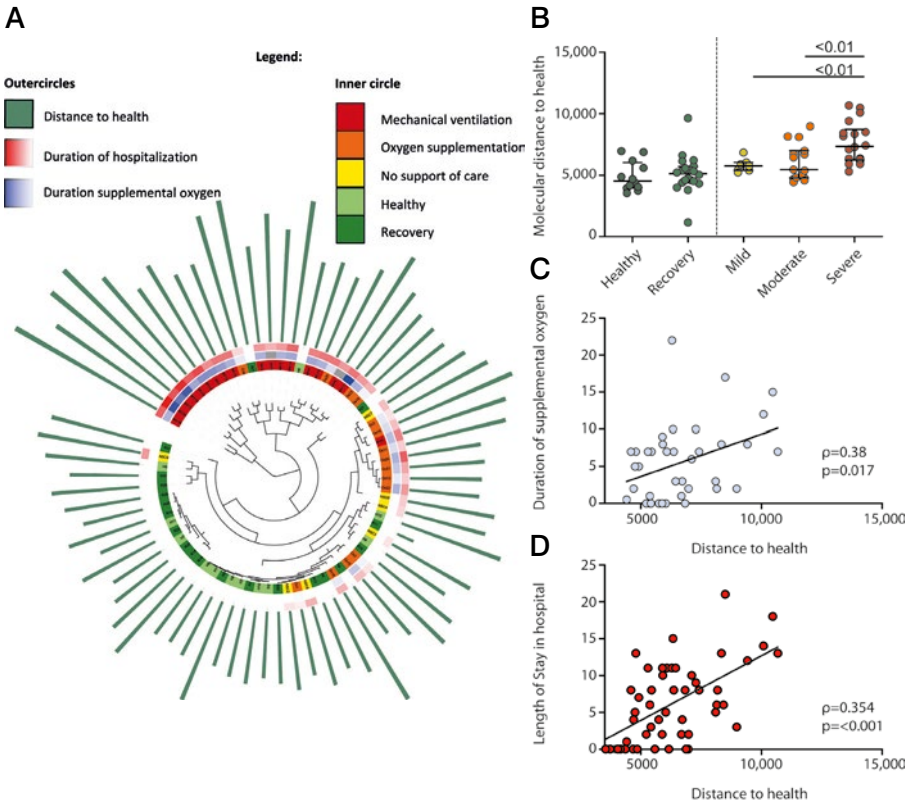

Figure 11.1 Patients with viral lower respiratory tract infections cluster together on disease severity. **A.** The distance to health score of individual patients was used to create this graph. The outer green circle depicts the DTH scores of individual patients. With Euclidian clustering analysis the patients clustered based on disease severity, as reflected by the inner part of the figure. Further, duration of hospitalization and supplemental oxygen are shown in the middle circles of the graph. **B.** The DTH scores are shown per severity group, **C.** Correlation of DTH with duration of supplemental oxygen and **D**. length of stay in the hospital (spearman correlation).

platelets and erythrocytes. This method also revealed significant differences in the modules apoptosis/survival, plasma cells, mitochondrial stress, cell death and B cells **(Supplemental figure 2)**.

The changes in the specific modules during acute, and especially severe disease led us to further explore the immunological processes that underlie this difference in gene expression. Downregulation of the cytotoxic T cell/ NK cell, and T cell module may be explained by a decreased cell count of these specific cell types, as has been published

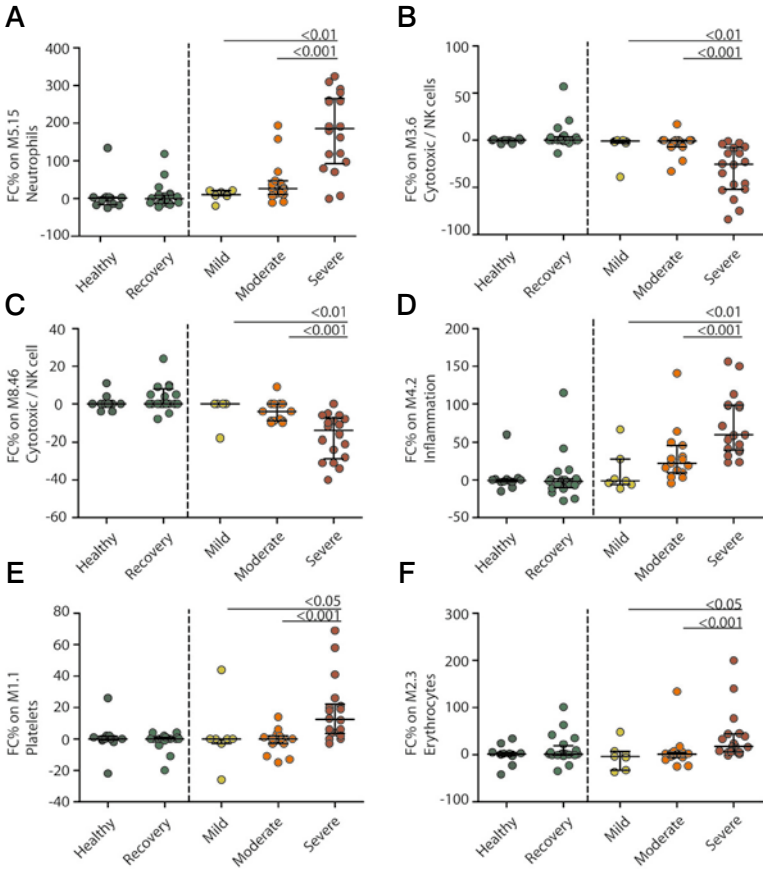

Figure 11.2 A-F Individual scores per severity group on modules that are differently expressed during acute disease. Additional information on the modules **A**. Neutrophil modules: healthy and recovery values are similar, all scores are higher during acute disease (healthy vs mild $p<0.05$, healthy vs moderate / severe $p<0.01$ and healthy vs severe $p<0.001$). **B**. Cytotoxic T cell / NK cell module: the scores of the healthy, recovery, mild and moderate group are similar. The severe group has a lower score compared to the healthy and recovery group ($p<0.0001$). **C**. Cytotoxic T cell / NK cell module: the scores of the healthy, recovery and mild group are similar. Healthy vs moderate $p<0.05$, severe vs healthy and recovery group $p<0.0001$. **D.** Inflammation module: Healthy and recovery and mild scores are similar, healthy vs moderate $p<0.001$ and severe $p<0.0001$. **E.** Platelets modules, healthy and recovery, mild and moderate patients have similar scores, healthy infants have significantly lower scores compared to severe diseased patients ($p<0.01$). **F.** Erythrocyte module, scores between healthy and recovery, mild and moderate are similar. Healthy vs severe $p<0.01$.

before by our group and others [25,26]. Therefore, in this study these specific cell populations were not further explored. Also erythrocytes and platelets were not studied, since the required materials were not collected during the original study. We therefore choose to focus on the inflammatory response and neutrophils in this study.

Cytokine and chemokines measurements as validation of the inflammation module

Plasma was stored from the majority of patients and the levels of ten different proteins related to inflammation were measured. Interleukin-6, Interleukin-8, Interleukin-1β, IL-10, gCSF, RANTES and MCP-1 levels were statistically significantly lower in the healthy controls than in the RSV infected groups (**Supplemental figure 3**). For 27 patients both CBA and microarray data were available and a significant differences in IL-6, and RANTES concentrations among the three severity groups was found. RANTES correlated with DTH and the duration of supplemental oxygen (Pearson rho -0.45, $p < 0.001$ and -0.43, $p < 0.05$, respectively).

Neutrophil specific microarray

To see whether the upregulation of the neutrophil module is due to the influx of a new population of young and banded neutrophils or activation of normal neutrophils we used a dataset published by de Kleijn *et al.* [23]. In this study gene expression studies were performed on subsets of neutrophils, e.g. normal, activated and young neutrophils, derived from volunteers 4 hours after LPS infusion. Two sets of genes were generated, the first comprised of differentially expressed genes when normal neutrophils are compared to activated neutrophils **(figure 3A)**, and the second comprised of genes derived from the comparison of normal versus young neutrophils **(Figure 3B)**. Genes from these lists were subsequently used on gene expression data from neutrophils of RSV infected infants. Clustering revealed no differences between the samples based on the activated neutrophils gene set (**Figure 3C)**. However, the set based on young neutrophils enabled clustering of patients **(Figure 3D).**

Differences in neutrophil subsets as explanation for differences in gene expression in the neutrophil module

The highly differentially expressed neutrophil modules in patients with a severe course of disease may be caused by differences in absolute cell counts, as is the case for the downregulation of the cytotoxic T cell and NK module or, as indicated by the neutrophil micro-array, an influx of young neutrophils recruited from the bone marrow. Therefore, blood smears of the patients were analyzed. No differences were seen in absolute lymphocyte and leukocyte counts between the severity groups (**Figure 4A and B**). Neutrophil counts were increased in the severe group compared to the mildly diseased patients. The median of the moderate group was even higher than that of the severe group, but due to the large range of values no significance was reached (median of 0.51, 2.62 and 2.15 $*10^6$ neutrophils/ml during mild, moderate and severe disease,

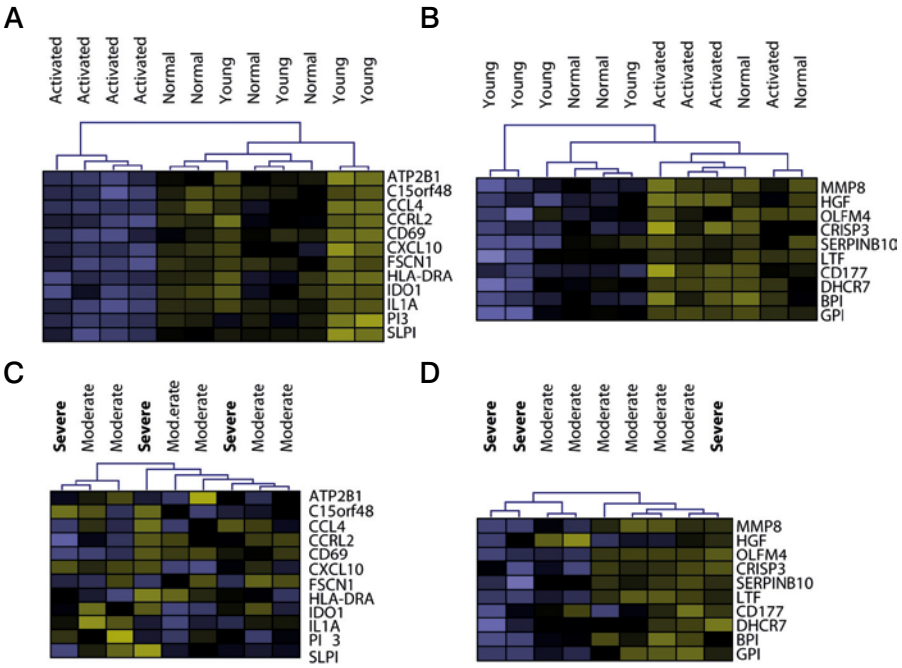

Figure 11.3 Gene list of most differentially expressed genes in activated (**A**) and young (**B**) neutrophils derived from LPS infused volunteers, are applied to neutrophils of RSV infected infants. **C.** No clustering of severe patients with the gene list of activated neutrophils. **D** Genelist of young neutrophils, two severely ill patients cluster together with 2 moderate patients. Purple represents upregulation and green downregulation.

respectively) (**Figure 4C**). The number of banded neutrophils was significantly higher in patients with a severe course of disease (**Figure 4D**). The number of banded neutrophils correlated with the score on the neutrophil module, $\rho=0.57$, $p<0.001$.

Gene expression differences based on subset of neutrophils

Thus far, we showed that the neutrophil module is highly differentially expressed in patients with a severe course of disease. This can be explained by the influx of young, banded neutrophils, as reflected by the differences in cell counts and results of the neutrophil gene expression. However, these small differences can therefore only partly explain this observation. From some patients also gene expression data of the PBMC fraction was obtained and it was checked whether the activation and young neutrophil gene lists from the LPS study could enable clustering within these PBMC. No clustering was seen in the expression of genes associated with activated neutrophils (**Figure 5A)**. In contrast, the expression of genes associated with young neutrophils showed

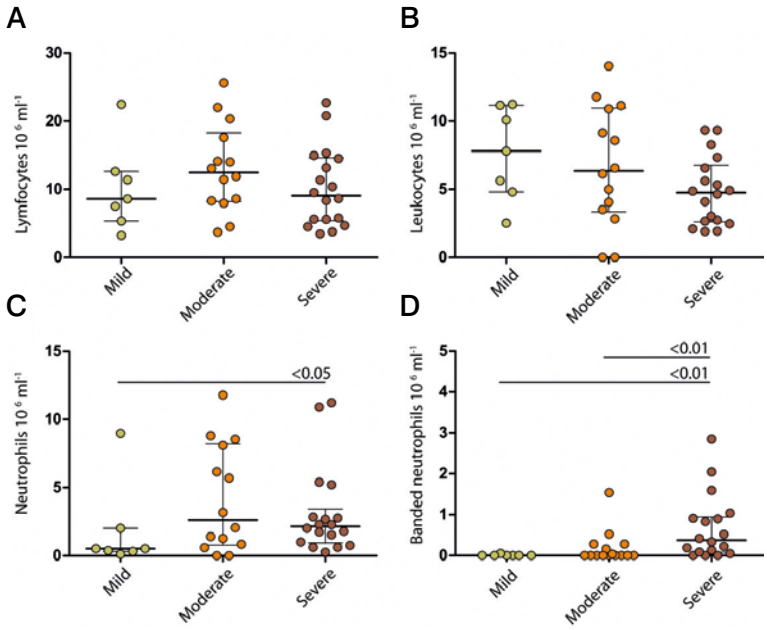

Figure 11.4 Cell differentiation of the blood of the same patients as used for the micro-array. Cell differentiations of circulating blood cells in patients with RSV infections with different disease severities. Data are represented as amount per milliliter blood, with median and interquartile range, Mann Whitney U was used to test for differences between two groups.

increased expression of all genes in severe disease, whilst the mild and moderately ill patients had no or low expression of these genes (**Figure 5B**).

PBMC obtained during acute RSV infection reveals the presence of neutrophils

To explore the phenomenon of a neutrophil signature in PBMC, FACS analysis was performed on stored PBMC of infants with acute RSV infection to determine the presence of neutrophils. Based on the forward and sideward scatter and expression of CD15, the presence of 2,0 - 7,1 % CD15+ cells which are most likely neutrophils with a lower density in the PBMC fraction could be confirmed.

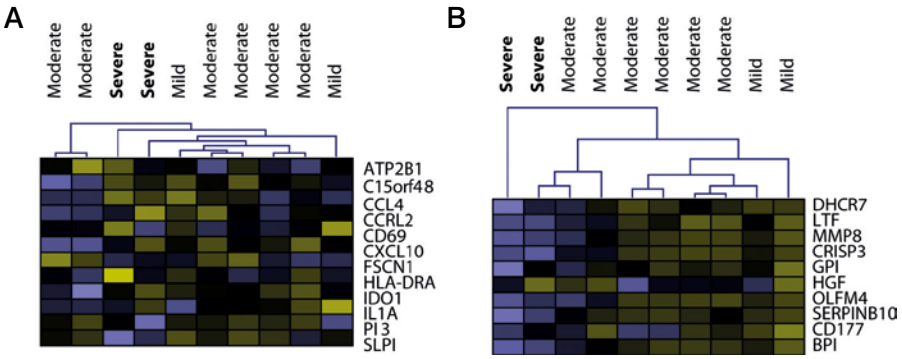

Figure 11.5 Gene list of neutrophils from LPS study based on activated (**A**) or young (**B**) neutrophils used in PBMC of RSV infected infants. **A** No clustering of patients when the gene list of activated neutrophils is applied to gene expression data of PBMC of RSV infected infants. **B**. The severely ill patients cluster together with 2 moderately ill patients based on the gene list of young neutrophils. Purple represents upregulation and green downregulation.

Discussion

In this study we analyzed gene expression profiles of infants with difference in levels of disease severity due to RSV infections to gain insight in the pathophysiological processes. We show that especially expression of genes related to the neutrophil module are strongly upregulated during severe RSV infection. Severe disease was also associated with downregulation of modules related to cytotoxic T, NK and B cells and upregulation of modules related to inflammation, platelets and erythrocytes. Upregulation of genes in the neutrophil module was further explored and we show that the response seems to be correlated with an influx of young neutrophils into the peripheral blood, and the appearance of a neutrophil signature in the lower density PBMC fraction.

Clinically, the correlation of RSV infections with neutrophil responses has been demonstrated before, although this is not fully understood yet. A strong influx of neutrophils into the blood occurs at the peak of the clinical symptoms in infants with an RSV infection. [27-29] A study by Lukens *et al.* on sequentially sampled patients with RSV infections showed a systemic neutrophil response with an increase of bone marrow-derived neutrophil progenitor cells during symptomatic disease.[27] Also at the site of infection neutrophils counts increase, reflected by the presence of neutrophils up to 80% in bronchoalveolar lavages of patients with severe RSV infections requiring mechanical ventilation.[29-32] In non-ventilated children with severe RSV infections this neutrophil influx has been described as well, diminishing the likelihood of a ventilator induced phenomenon.[26] Neutrophils in the lungs of RSV infected patients are activated, as they show delayed apoptosis and release cytokines.[33,34] To date, it has not been shown that neutrophils

have a direct role in the anti-viral host response.[35] However, RSV seems to have adapted to the neutrophils since it can bind to and transcribe itself in neutrophils in the peripheral blood resulting in neutrophils that express RSV proteins on their surface. [36]
A specific population of neutrophils, that was found to co-purify with the PBMC fraction after density gradient centrifugation has been described in the context of auto-immunity. In 1986, Hacbarth et al. described these cells in patients with systemic lupus erythematosus (SLE) and named them low density granulocytes (LDG).[37] The first publications on different densities of neutrophils appeared already in the seventies, where the granulocyte fraction after density gradient centrifugation was further subtyped using Percoll density gradient.[38] The percentage of lower density neutrophils in this fraction increased during sepsis or bacterial infections.[39] These 'low-buoyant' density granulocytes appeared to have emptied their granulae as was shown with electron microscopy and have diminished chemotactic capacities. [39] This neutrophil subpopulation was found to be pro-inflammatory. Moreover, these cells are easily triggered to form neutrophil extracellular traps.[40] Further characterization of the LDG appear to be contradictory. Based on gene expression an immature granulocyte is expected, whilst a flow cytometry study showed that the LDG population resembles mature granulocytes which express low CD14 and high CD15.[41] Microscopy studies on the nuclear morphology of these cells describe a mixed population of young and mature granulocytes. The origin of this neutrophil subset is still unknown.[40] Besides literature on low density granulocytes, studies on granulocytic myeloid derived suppressor cells (G-MDSC's) are also emerging. The G-MDSC is a heterogeneous subset of cells that expands during inflammation, infections and cancer and suppress T cell responses.[42] This cell type is a likely candidate for our neutrophil signature in the PBMC fraction, since it also resides in the PBMC fraction after density gradient centrifugation and it is described in a wider range of immunological processes. Moreover, they closely resemble neutrophils, differences and similarities between G-MDSC and neutrophils are reviewed extensively by Pillay *et al.* [43]. As was seen in LDG's, the phenotype of G-MDSC's ranges from immature to mature and all types share distinct features of the neutrophil e.g. nucleus morphology and surface markers. In this study, we used gene expression data from subsets of neutrophils based on surface markers to increase our insight in the composition of the neutrophil fraction in the PBMC. In a follow up study on the neutrophil subsets described by de Kleijn *et al*, P2 neutrophils (activated neutrophils or $CD62L^{dim}/CD16^{bright}$) were found to be immune suppressive. It is tempting to speculate that G-MDSC's are not the cell type that is responsible for the upregulation of the neutrophil module, since there was no clustering of severe patients in the PBMC micro-array when the P2 list was used. However gene expression data might not be the most optimal tool to pinpoint this specific cell population since gene expression, microscopic and flow cytometry data have proven to be contradictory in both LDG and G-MDSC's. We have shown that our population has an immature gene expression profile and is $CD15^{+}$ representing a form of innate immunity.[23]

The neutrophil module upregulation may be related to several other factors. Bacterial co-infection or mechanical ventilation should also be considered as trigger for the immune response. The group of Ramilo *et al.* recently published a large study on infants with different viral respiratory infections, including RSV. [44] They also used a modular approach and showed similar results concerning disease severity in different cohorts of infants. We were able to validate their findings concerning the upregulation of specific modules during severe RSV infections in infants, on a different microarray platform. Although both groups exclude patients with (suspected) bacterial co-infections many of them received antibiotics, limiting the value of negative blood cultures. We reviewed the clinical charts of patients with a high score on the neutrophil module and made two important observations. First of all, not all patients with high neutrophil scores received mechanical ventilation or received antibiotics during their treatment. Second, patients with a high neutrophil score without the need for mechanical ventilation, did experience multiple apneas during their hospitalization. This finding indicate that the activation of the neutrophil module is not explained by a reaction towards a bacterial co-infection or mechanical ventilation only. It would be interesting to investigate whether there is a relation between neutrophil activation and the incidence of apnea in infants with RSV, because it has been shown that the occurrence of OSAS (obstructive sleep apnea) is correlated with neutrophilic inflammation of the lower airways. [45]

The increased expression of genes of the inflammation module as observed in this study is in-line with other studies which showed increased cytokine and chemokine levels during viral infections. [25,28] IL-6 and RANTES levels were significantly different among the severity groups and IL-8 correlated with disease severity measures as described before. [28] The downregulation of the NK and cytotoxic T cell module in patients with severe RSV infections is in line with other studies that show lower cell counts of T cells and NK cells during severe RSV infections. [25,26] The platelet and erythrocyte modules are upregulated during viral infections. Both cell types have immune-modulatory capacities that are only now being recognized as important players in the immune system and immunological studies on these cell types start to emerge. Activated platelets can influence the cytokine response of monocytes upon stimulation with LPS, resulting in a decreased TNF response and increased IL-10 production. [46] The immune-modulatory effects of erythrocytes has been revealed by Elahi *et al.* who showed that neonatal erythroids are rendering the neonatal immune system hypo responsive and thereby contribute to neonatal susceptibility to infections. [47]

Bont *et al.* conclude in their study that mechanical ventilation and hyperoxia results in an increased inflammatory response in the lungs of RSV infected patients. [48] However, an increase in the neutrophil and inflammation modules was also observe and mechanical ventilation and course of disease. Therefore, we think that mechanical ventilator or hyperoxia induced damage is not likely to be a major confounder.[49]

Although our study has a limited sample size, our findings are in accordance with a completely independent and recent study from Mejias et al.[44] For future studies sequential data on the immune response of infants during RSV infection would increase

insight in the pathophysiology of the host response of severe disease. Increasing numbers of healthy controls and mildly ill patients would further increase the discriminative power of the used analysis methods. In summary, this study provides new insights into severe RSV infections by the use of micro-array analysis and indicate that neutrophils, cytotoxic T-cells, NK-cells, platelets and erythrocytes play a role in the pathophysiology of severe disease. A population of neutrophils, most likely LDG's or G-MDSC's, appeared in the PBMC fraction of patients with a severe course of their RSV infection. These findings open new avenues for further research on the pathophysiology of RSV induced disease and could enable the development of a prognostic tool to predict the course of infection.

Acknowledgements

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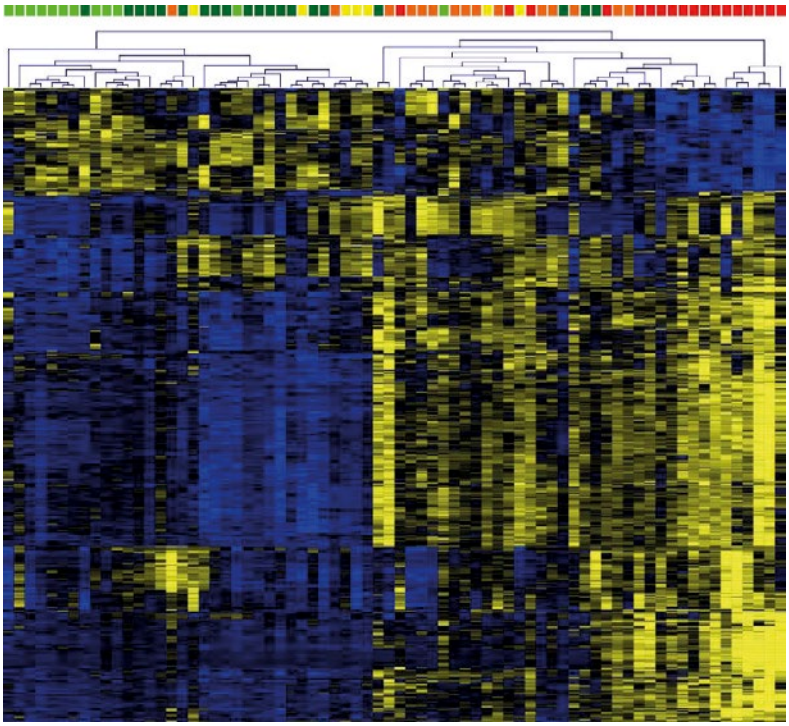
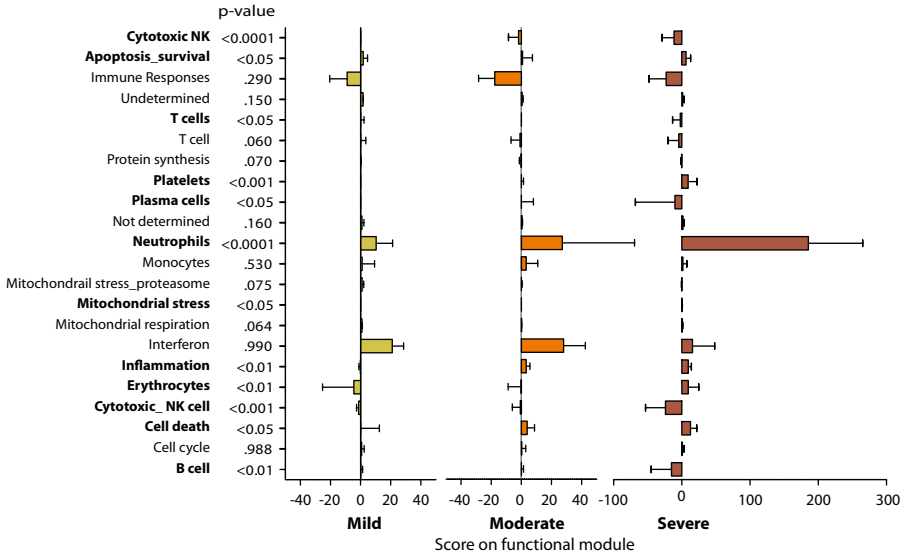
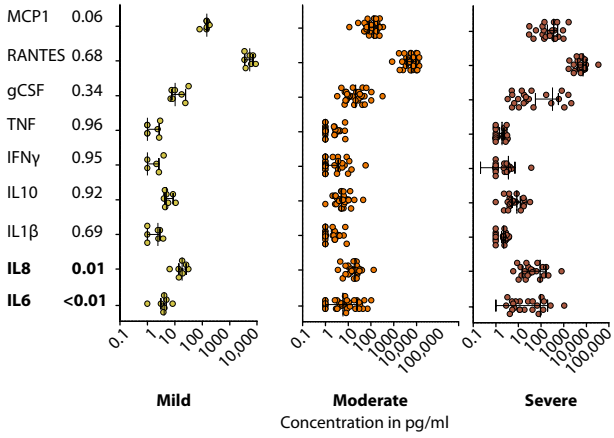


Supplemental Figure 1 Heat map of significantly changed genes between the individual patients and the control group.



Supplemental Figure 2 Median scores on functional modules per severity group. Twelve out of 27 functional modules changed in any of the three severity groups (Kruskal Wallis).



Supplemental Figure 3 Plasma levels of cytokines and chemokines in healthy infants, recovery samples and infants with different severities of an RSV infection. Plasma levels of 11 healthy, 27 recovery, 7 mild, 39 moderate and 22 severe patients are depicted with median and interquartile ranges, samples below the detection limit were set to 1 to enable visualization. Significance is calculated among the mild, moderate and severe group.

12

Nasopharyngeal gene expression, a novel approach to study the course of respiratory syncytial virus infection.

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Abstract

Respiratory syncytial virus (RSV) causes mild infections in the vast majority of children. However, in some cases, it causes severe disease, such as bronchiolitis and pneumonia. Development of severe RSV infection is determined by the host response. Therefore, the main aim of this study was to identify biomarkers associated with severe RSV infection.

To identify biomarkers, nasopharyngeal gene expression was profiled by microarray studies, resulting in the selection of five genes: *ubiquitin D*, *tetraspanin 8*, *mucin 13*, β-*microseminoprotein* and *chemokine ligand 7*. These genes were validated by real-time quantitative PCR in an independent validation cohort, which confirmed significant differences in gene expression between mildly and severely infected and between recovery and acute patients.

Nasopharyngeal aspirate samples are regularly taken when a viral respiratory tract infection is suspected. In this article, we describe a method to discriminate between mild and severe RSV infection based on differential host gene expression. The combination of pathogen detection and host gene expression analysis in nasopharyngeal aspirates will significantly improve the diagnosis and prognosis of respiratory tract infections.

Introduction

Respiratory syncytial virus (RSV) is the most common cause of viral respiratory tract infections among hospitalised children. Symptoms range from a common cold to severely compromised respiratory function, causing 6-15% of the admitted children to require intensive care[1,2]. It is difficult to predict the course of disease in infants, especially in children under the age of 3 months. Current clinical prediction rules are based on demographic criteria and clinical symptoms[3-10] and although they may improve clinical judgement[7,8], they are not routinely used in daily practice. More objective and reproducible predictions may be achieved by using biomarkers[11-15].

Recent publications showed that gene expression profiles in peripheral blood mononuclear cells represent the subject's health. Ramilo *et al.*[16] were able to discriminate influenza A, *Escherichia coli* and *Streptococcus pneumoniae* infection with 95% accuracy, and distinguished *E. coli* from *Staphylococcus aureus* infection with 85% accuracy by analysing mRNA expression level in young patients. In the case of RSV infections, Mejias *et al.*[17] showed, based on blood RNA profiles of infants, a classification of infants with RSV lower respiratory tract infection (LRTI) versus rhinovirus or influenza LRTI with 95% accuracy. Additional studies confirmed these observations and described the discovery of several biomarkers in blood by which different states of disease could be distinguished[18].

These findings may be of great value for clinical decision making. However, the studies, as described, were performed using blood samples, of which only limited volumes can be obtained from paediatric patients. Thus, it would be worthwhile to study whether other clinical materials could be used that reflect the host response and may harbour prognostic value. The nasopharynx is the point of entry of all respiratory viruses; moreover, a sample from this site is already taken to diagnose viral infections using real-time quantitative PCR (RT-qPCR)[19]. The use of nasopharyngeal aspirates (NPAs) for the measurement of host biomarker expression for prognostic purposes would be worthwhile to assess, especially as clinical laboratories would only need minimal adjustment to integrate this test into current practice. Knowledge in regards gene expression in the nasopharynx during RSV infections is limited and has been gained solely from animal models[20].

In this study, we assessed the possibility of measuring host gene expression in NPA taken from children with laboratory-confirmed RSV infections and aimed to identify markers with potential prognostic value for the course of disease.

Materials and methods

Ethics. The study was approved by the Committee on Research Involving Human Subjects of the Radboud University Medical Center (Nijmegen, the Netherlands). All clinical samples collected from patients in our cohorts were enrolled following informed

consent from parents or guardians. The clinical information available to subjects being recruited for the study included a clear description of the risks and benefits of participation, indicating the required insurance and procedures, safe handling and protection of all data. All subjects were informed that there was no obligation to participate and that declining to participate or leaving the study had no adverse consequences.

Study design. Children <5 years of age with symptoms of viral LRTI were included. These symptoms included increased respiratory effort (e.g. tachypnoea and/or use of accessory respiratory muscles) and/or expiratory wheezing and/or crackles and/or apnoea. Retrospectively, the LRTI was confirmed by RT-qPCR on nasopharyngeal washes as described previously[19]. Patients with congenital or acquired immune deficiency, immunosuppressive medication (including >24 hours of glucocorticosteroids) or severe psychomotor retardation were excluded.
All subjects were recruited at two hospitals in Nijmegen. Subjects admitted were screened for symptoms typical for viral respiratory tract infection, as described previously. When RSV infection was suspected, a NPA was collected within 24 hours of admission (acute) and parents of hospitalised children were asked permission to draw a second NPA sample 4–6 weeks after admission (recovery). Medical history, demographics and clinical parameters were collected from questionnaires and medical records. Patients were classified into three different groups based on the degree of supportive care they needed during the course of disease: no supportive care; supplemental oxygen (when oxygen saturation was <93% for >10 minutes); or mechanical ventilation.
Microarrays were performed on samples from the cohort published by Brand *et al.*[18] (n=30, 2006–2009) and subsequently selected markers were validated in the cohort of I.M.L. Ahout and co-workers (personal communication; manuscript in preparation) (n=44, 2010–2012).

Sample collection. After informed consent, a NPA sample was collected by introducing a catheter, connected to a collection tube and an aspiration system, into the nasopharyngeal cavity. Then, saline (0.5-1.5 mL) was instilled into the catheter and, while slowly retracting the catheter, the nasopharyngeal fluid was aspirated in a collection tube. Afterwards, the catheter was flushed with saline and added to the collection fluid.
The samples were kept on ice and immediately transferred to the laboratory, where they were centrifuged (500×g) for 10 minutes at 4°C to spin down the mucus and cells, after which the pellet was mixed with Trizol (Life Technologies, Carlsbad, CA, USA) and stored at -80°C. RT-qPCR was performed using the supernatant to confirm viral aetiology of the disease[19].

Microarray. RNA was extracted from NPA in Trizol according to the manufacturer's protocol. The final RNA pellet was washed twice with 1 mL of 75% ethanol, air dried and resuspended in 100 μL of RNAse/DNase free water. Subsequently, a clean-up of the total RNA was performed with the RNeasy Minikit (Qiagen, Hilden, Germany) according

to the manufacturer's instructions. RNA integrity and quality for the microarray was assessed using a RNA 6000 Nano LabChip on an Bioanalyzer 2100 system (Agilent, Santa Clara, CA, USA).
RNA processing, target labelling and hybridisation to gene expression arrays was performed. Biotin-labelled cRNA was obtained using the One-Cycle Eukaryotic Target Labeling Assay (Affymetrix Inc., Santa Clara, CA). 15 μg of fragmented, biotin-labelled cRNA was hybridised to Affymetrix GeneChip Human Genome U133 Plus 2.0 arrays according to standard Affymetrix protocol.

Microarray data analysis. Quality control analyses were performed as previously described[21]. Scanned images were inspected for artefacts, percentage of present calls (<25%) and controls of RNA degradation. Background signal was removed using robust multichip analysis and probe intensity levels were quantile-normalised across arrays. Microarray analysis was performed using ArrayStar4 (DNAStar, Madison, WI, USA).
After the microarray data were obtained, comparisons were made based on supportive care: the need for supportive care (comparison 1) and mechanical ventilation (comparison 2). *p*-values were corrected for false discovery rate and, subsequently, the significant genes with a four-fold change were selected, while probes directed at noncoding sequences were excluded[22].
The selected genes were not normally distributed according to the d'Agostino and Pearson omnibus normality test (α=0.05) (Prism 5; Graphpad Software, La Jolla, CA, USA). Therefore, a permutation test using MultiExperiment Viewer (tm4group; Dana-Farber Cancer Institute, Boston, MA, USA) with random group samples (10^5) was performed and the median fold change cut-off was set at 2.50 log. The overlapping genes between the two comparisons and the only upregulated gene were selected for validation by RT-qPCR.

Gene validation by RT-qPCR. Samples were defrosted on ice and RNA was extracted from NPA in Trizol according to the manufacturer's protocol. The RNA pellet was washed twice with 1 mL of 75% ethanol, air dried, re-suspended in 100 μL of diethylpyrocarbonate-treated water. Subsequently, a clean-up was performed with the RNeasy Minikit according to the manufacturer's instructions. The elutate was treated with TURBO DNA-free (Life Technologies); subsequently, cDNA synthesised by iScript (Biorad, Hercules, CA, USA) and stored at -20°C.
The RT-qPCR was conducted on a CFX96 (Biorad) using Taqman gene expression assays (Hs.00197374_m1, Hs.00610327_m1, Hs.00217239_m1, Hs.00738231_m1, Hs.00171147 _m1 and Hs.02758991_g1; Life Technologies) and IQ Powermix (Biorad). The PCR programme consisted of an initial 5 minutes at 96°C, followed by 40 cycles of 15 seconds at 96°C and 45 seconds at 60°C. The data generated were analysed using CFX Manager 3.0 (Biorad) and the cut-off value was set by using the single threshold mode for quantification cycle (Cq) determination. The data were normalised by subtracting the Cq-value of the housekeeping gene, i.e. *glyceraldehyde 3-phosphate dehydrogenase* (*GAPDH*) (Hs.02758991_g1; Life Technologies).

Statistical analysis. Significance of the microarray analysis was calculated by a permutation test, with 10^5 random group samples using TMEV (tm4group). Significance of the RT-qPCR-measured gene expression was calculated by Mann–Whitney U-tests using Prism 5, where a two-sided value of $p<0.05$ was considered statistically significant. Correlation was assessed by Spearman analysis using SPSS (IBM, Armonk, NY, USA), where a two-sided value of $p<0.05$ was considered statistically significant. For the patient characteristics, the Kruskal–Wallis analysis was performed; in the case of significance ($p<0.05$), we subsequently performed Mann-Whitney U-tests for individual comparisons using SPSS.

Results

Microarray data analysis. In this study, the clinical value of nasopharyngeal gene expression was assessed by conducting microarrays on NPAs of 30 infants with a confirmed RSV infection, both mono- (RSV) and co-infections. The patient characteristics are displayed in table 1. No significant differences were found, except for the parameters on which the clinical categorisation was based and gestational age.

To select potential biomarkers, comparisons were made using microarray analysis with regards to the need for supportive care: supplemental oxygen (comparison 1) and the need for mechanical ventilation (comparison 2). The permutation test led to an initial selection of 53 targets (table S1) for comparison 1 and 93 targets for comparison 2 (table S2). By the subsequent introduction of a median fold change cut-off of 2.5 log, 16 genes were selected that were significantly differentially expressed in comparison 1 (table S3) and 28 genes were selected in comparison 2 (table S4).

The two selections were compared and four overlapping genes were found (*ubiquitin D* (*UBD*), *tetraspanin 8* (*TSPAN8*), *mucin 13* (*MUC13*) and β-*microseminoprotein* (*MSP*)), which all showed downregulation. In addition to these genes, *chemokine ligand 7* (*CCL7*) was selected as the only upregulated differentially expressed gene in the whole microarray analysis (table 2).

Gene expression validation. In order to confirm differential expression of the genes selected by microarray analysis, RT-qPCR was used to validate these genes in an independent cohort. This cohort consisted of patients with RSV mono-infections to focus on the RSV specificity of the discovered genes. The final cohort consisted of 42 RSV-infected infants, of whom the patient characteristics are displayed in table 3. No significant differences were found between the groups, except for the parameters on which the clinical categorisation was based, age and RSV serotype.

To assess the overall sample quality, the expression of *GAPDH* in all samples was plotted (figure 1). Generally, the amounts of RNA in the NPA samples were low; in a few samples, the amounts were too low to be able to measure gene expression, so these samples were excluded from further analysis.

Table 12.1 Microarray cohort patient characteristics.

	No supportive care	Supplemental oxygen	Mechanical ventilation	*p*-value	
				Kruskal–Wallis test	*Mann–Whitney U-test*
Patients [n]	10	10	10		
Age [days]	227 (57–279)	135 (46–337)	64 (41–161)	NS	NA
Males [n (%)]	8 (80)	8 (80)	8 (80)	NS	NA
Gestational age [weeks]	40 (37–41)	38 (35–40)	33 (33–35)	<0.01**,##	<0.01¶¶,+
RSV load [Cq-value]	31 (25–33)	31(24–33)	31 (28–32)	NS	NA
Duration of symptoms [days]	7 (4–9)	5 (3–7)	5 (4–5)	NS	NA
Duration of oxygen therapy [days]	0	3 (1–6)	9 (5–11)	<0.001***,###	<0.001¶¶¶,++,§§§
Length of hospital stay [days]	0 (0–3)	5 (2–7)	13 (7–16)	<0.001***,###	<0.001¶¶¶,++,§

Data are presented as median (interquartile range), unless otherwise stated. RSV: respiratory syncytial virus; Cq-value: quantification cycle value; NS: nonsignificant; NA: not applicable. **: $p<0.01$ for no supportive care *versus* supportive care (supplemental oxygen or mechanical ventilation); ***: $p<0.001$ for no supportive care *versus* supportive care; ##: $p<0.01$ for no mechanical ventilation (no supportive care or supplemental oxygen only) *versus* mechanical ventilation; ###: $p<0.001$ for no mechanical ventilation *versus* mechanical ventilation; ¶¶: $p<0.01$ for no supportive care *versus* mechanical ventilation; ¶¶¶: $p<0.001$ for no supportive care *versus* mechanical ventilation; +: $p<0.05$ for supplemental oxygen *versus* mechanical ventilation; ++: $p<0.01$ for supplemental oxygen *versus* mechanical ventilation; §: $p<0.05$ for no supportive care *versus* supplemental oxygen; §§§: $p<0.001$ for no supportive care *versus* supplemental oxygen.

Table 12.2 Microarray cohort based gene selection.

Gene	Gene product	Supportive care		Mechanical ventilation		TaqMan gene expression assay
		p-value	*Median fold change*	*p-value*	*Median fold change*	
UBD	Ubiquitin D	0.00271	−3.50	0.00004	−3.54	Hs.00197374_m1
TSPAN8	Tetraspanin 8	0.00429	−3.39	0.00157	−2.81	Hs.00610327_m1
MUC13	Mucin 13	0.00593	−3.54	0.00002	−3.41	Hs.00217239_m1
MSP#	β-microseminoprotein	0.00168	−4.09	0.00009	−2.83	Hs.00738231_m1
CCL7	Chemokine ligand 7	NA	NA	0.00061	2.70	Hs.00171147_m1

Na: not applicable. #: multiple probes were found; the average *p*-value and median fold change are displayed.

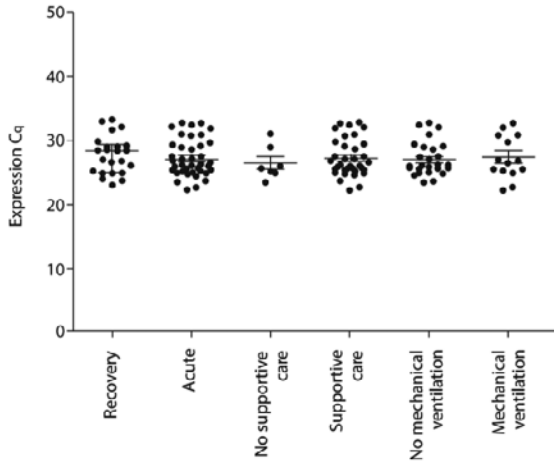

Figure 12.1 Nasopharyngeal expression of *glyceraldehyde 3-phosphate dehydrogenase*. Data are presented as median and interquartile range. Data were Mann-Whitney U-tested, two-sided (recovery, n=23; acute, n=42). No significant differences were found. Cq: quantification cycle.

These results clearly show a low but consistent expression in all samples, irrespective of the patient's health status. RT-qPCR analysis of our selected genes using nasopharyngeal samples from the validation cohort confirmed that the expression of all selected genes was significantly different, when comparing the recovery with acute samples. In this analysis, the acute group consisted of pooled expression data from all three groups: no supportive care, supplemental oxygen and mechanical ventilation. In case of *TSPAN8*, *MUC13*, *MSP* and *CCL7*, the expression differed significantly in comparison 2 (the need for mechanical ventilation) (figure 2). Subsequently, the correlation between the level of gene expression and known risk factors was assessed. Smoking at home, smoking during pregnancy, eczema, asthma and atopic family history did not show any correlation. For *UBD* only, a correlation with gestational age was found; however, gestational age is a well-known confounder, which does not exclude the value of *UBD* as severity marker[23].

Table 12.3 Validation cohort patient characteristics.

	No supportive care	Supplemental oxygen	Mechanical ventilation	*p*-value	
				Kruskal–Wallis test	*Mann–Whitney U-test*
Patients [n]	7	21	14		
Age [days]	153 (102–325)	74 (55–179)	42 (28–76)	<0.05*,#	<0.01++
Males [n (%)]	5 (71)	10 (48)	7 (50)	NS	NA
Gestational age [weeks]	39 (35–40)	39 (37–40)	39 (37–40)	NS	NA
RSV load [Cq-value]	22 (21–22)	25 (22–29)	24 (21–26)	NS	NA
RSV serotype	6 (86)	11 (52)	14 (100)	<0.01##	<0.05§
Duration of symptoms [days]	5 (4–5)	4 (3–5)	3 (2–5)	NS	NA
Duration of oxygen therapy [days]	0	3 (2–4)	9 (7–11)	<0.001***,###	<0.001¶¶¶,++,§§§
Length of hospital stay [days]	2 (0–6)	5 (4–8)	11 (10–13)	<0.001***,###	<0.001¶¶,+++,§§§

Data are presented as median (interquartile range) or n (%), unless otherwise stated. RSV: respiratory syncytial virus; Cq-value: quantification cycle value; NS: nonsignificant; NA: not applicable. *: $p<0.05$ for no supportive care *versus* supportive care (supplemental oxygen or mechanical ventilation); ***: $p<0.001$ for no supportive care *versus* supportive care; #: $p<0.05$ for no mechanical ventilation *versus* mechanical ventilation; ##: $p<0.01$ for no mechanical ventilation *versus* mechanical ventilation; ###: $p<0.001$ for no mechanical ventilation *versus* mechanical ventilation; ¶¶: $p<0.01$ for no supportive care *versus* supplemental oxygen; ¶¶¶: $p<0.001$ for no supportive care *versus* supplemental oxygen; ++: $p<0.01$ for no supportive care *versus* mechanical ventilation; +++: $p<0.001$ for no supportive care *versus* mechanical ventilation; §: $p<0.05$ for supplemental oxygen *versus* mechanical ventilation; §§§: $p<0.001$ for supplemental oxygen *versus* mechanical ventilation.

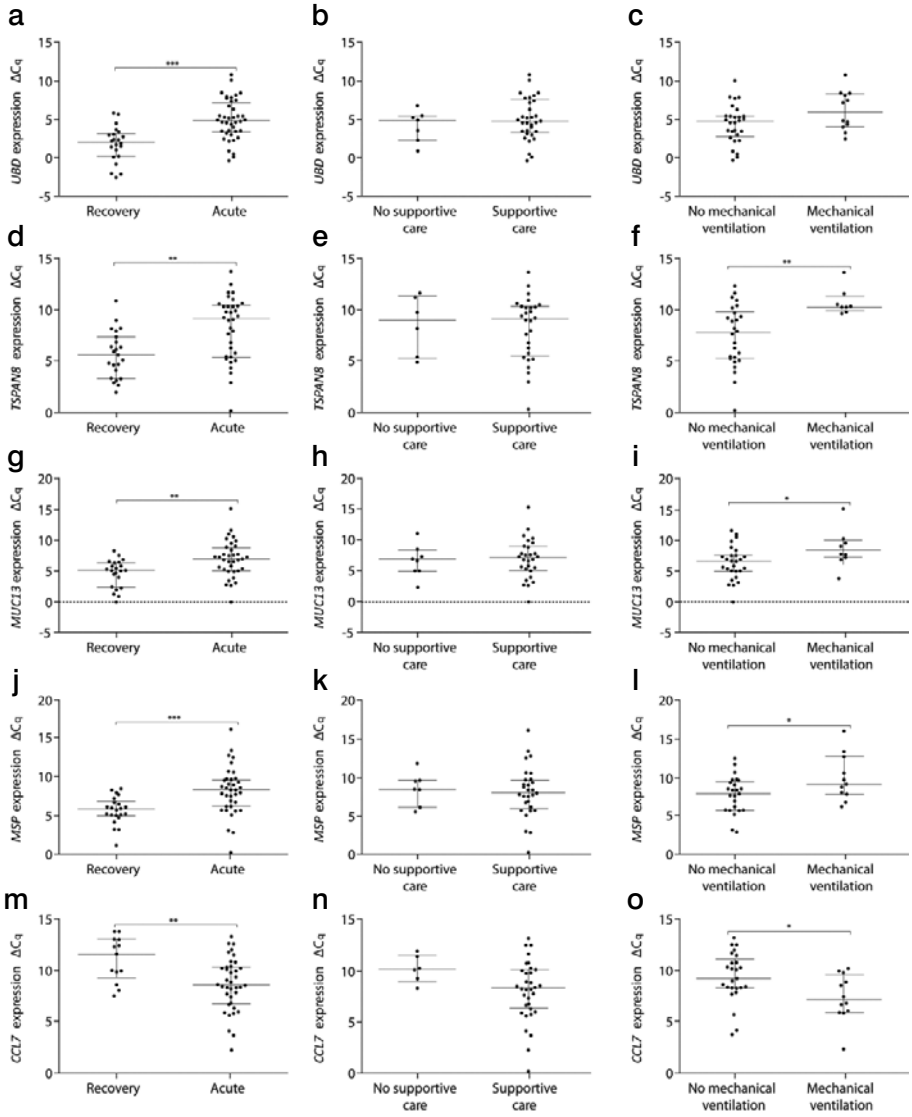

Figure 12.2 Nasopharyngeal expression of **a–c**) *ubiquitin D* (*UBD*), **d–f**) *tetraspanin 8* (*TSPAN8*), **g–i**) *mucin 13* (*MUC13*), **j–l**) β-*microseminoprotein* (*MSP*) and **m–o**) *chemokine ligand 7* (*CCL7*) in recovery *versus* acute (**a, d, g, j and m**), need for supportive care (**b, e, h, k and n**) and need for mechanical ventilation (**c, f, i, l and o**). Data are presented as median and interquartile range. Data were Mann-Whitney U-tested, two-sided (recovery, n=23; acute, n=42). ΔCq: *glyceraldehyde 3-phosphate dehydrogenase*-normalised quantification cycle. *: $p<0.05$; **: $p<0.01$; ***: $p<0.001$.

Discussion

This study shows that nasopharyngeal aspirates taken to diagnose the viral cause of infection can also be used to identify biomarkers by microarray analysis that are associated with severe RSV infections. The value of these markers was confirmed in an independent patient cohort. To our knowledge, this is the first study in which nasopharyngeal host gene expression was studied in the context of infection.

Gene expression analysis was unforeseen at the time of collection and a large part of the material was used for other studies; therefore, the quantity of the total material and the quality of the RNA isolated from the NPAs was suboptimal. The results of the *GAPDH* expression assay show that the absolute Cq-values are high, but consistent.

The microarray analysis resulted in the discovery of five significantly differentially expressed genes in the nasopharynx of infants with different degrees of severities of RSV infection. Remarkably, most genes are down-regulated in severely ill patients; this was confirmed with RT-qPCR. In addition, previously published microarray studies performed on gene expression profiles in peripheral blood of infants also showed a significant downregulation of genes[16,17].

The method, as described in this study, with the aim to identify genes with potential prognostic value, has proven successful by the validation of *TSPAN8*, *MUC13*, *MSP* and *CCL7*. Although UBD did not discriminate between any acute group, it differed significantly between the recovered and acutely ill patients. This clearly underlines the value of our approach, in which microarray analysis was used to select differentially expressed genes in a discovery cohort, which were independently validated by qPCR and RT-qPCR in a separate cohort.

Besides *CCL7*, none of the genes identified in this study has previously been described to be associated with RSV. *UBD* participates in the ubiquitination process, labelling proteins for their degradation via the proteasome. Mielech *et al.*[24] have shown that coronaviruses actively reduce ubiquitination of host cell proteins and observed their potential to modify the innate immune response. With regard to *TSPAN8*, Yue *et al.*[25] described its ability to override the adhesive features of CD151. On this note, CD151 is involved with the infectious entry of human papillomavirus[26], suggesting a significant infectious advantage for RSV when *TSPAN8* is downregulated. Reduced amounts of MUC13 lead to decreased chemokine secretion in response to tumour necrosis factor-α. This pro-inflammatory activity of MUC13 suggests that disrupted or inappropriate expression of *MUC13* could predispose to infectious disease[27]. The downregulation of *MSP* was previously described in the context of rhinovirus infection[28]. Mygatt *et al.*[29] reported virus-mediated silencing of *MSP*, which seems in accordance with our findings. Finally, significant upregulation of *CCL7* has been observed in mice infected with rhinovirus[20] and in children during infection by different respiratory viruses, including RSV[30]. In addition, *CCL7* was shown to be specifically upregulated when no mechanical ventilation was applied during RSV infection in mice and not differentially expressed when uninfected mice received mechanical ventilation[31], strongly suggesting

that *CCL7* upregulation is caused by RSV infection and is not a consequence of mechanical ventilation.
In conclusion, based on differential expression of *TSPAN8*, *MUC13*, *MSP* and *CCL7* in readily available NPA samples, we can discriminate between severity of disease in RSV infected infants. This proves the potential of additional host gene expression analyses using regularly taken NPA samples, which, when combined with pathogen detection, would improve diagnosis of respiratory tract infections. Therefore, our findings demand further studies that might lead to the implementation of nasopharyngeal gene expression in diagnostics to guide clinical decisions in the context of respiratory tract infections.

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Supplemental Table 12.1 Full Gene List based on Need for Supportive Care before 2.5 log cut-off.

Gene		Probe Set ID	*p-value*	Median fold change
RARRES3	Retinoic acid receptor responder 3	204070_at	0,00005	-2,11560
CLEC5A	C-type lectin domain family 5, member A	219890_at	0,00033	1,76341
VAMP8	Vesicle-associated membrane protein 8	202546_at	0,00081	-2,49187
ARG1	Arginase	206177_s_at	0,00153	2,49293
MSP	β-microseminoprotein	210297_s_at	0,00158	-3,83941
MUC5AC	Mucin 5AC	214385_s_at	0,00161	-2,44859
SYTL2	Synaptotagmin-like 2	232914_s_at	0,00177	-2,67463
MSP	β-microseminoprotein	207430_s_at	0,00178	-4,33859
UBD	Ubiquitin D	205890_s_at	0,00271	-3,50151
VMO1	Vitelline membrane outer layer 1	235751_s_at	0,00275	2,36093
CP	Ceruloplasmin	1558034_s_at	0,00312	-2,36217
HMGN3	High mobility group nucleosomal binding domain 3	209377_s_at	0,00373	-2,21560
RARRES1	Retinoic acid receptor responder 1	206391_at	0,00389	-2,13419
AGR3	Anterior gradient homolog 3	228241_at	0,00395	-2,47780
TSPAN8	Tetraspanin 8	203824_at	0,00429	-3,38764
NUCB2	Nucleobindin 2	203675_at	0,00433	-2,36256
IGLV1-44	Immunoglobulin lambda variable 1-44	214677_x_at	0,00464	-3,17083
Unannotated	n/a	235229_at	0,00521	-2,88441
CXCL10	Chemokine ligand 10	204533_at	0,00527	-1,35306
Unannotated	n/a	238103_at	0,00559	-2,85582
CP	Ceruloplasmin	227253_at	0,00568	-2,99898
C9orf135	n/a	243610_at	0,00582	-3,47416
MUC13	Mucin 13	218687_s_at	0,00593	-3,53651
GSTA1	Glutathione S-transferase alpha 1	203924_at	0,00620	-1,76308
TMC5	Transmembrane channel-like 5	240304_s_at	0,00653	-2,69625
NFIB	Nuclear factor I/B	209290_s_at	0,00729	-2,39278
PLUNC	Palate, lung and nasal epithelium associated	220542_s_at	0,00740	-1,87751

Supplemental Table 12.1 Continued.

Gene		Probe Set ID	*p-value*	Median fold change
SCGB1A1	Secretoglobin, family 1A, member 1	205725_at	0,00792	-2,94515
TFF1	Trefoil factor 1	205009_at	0,00847	-2,43802
TFF3	Trefoil factor 3	204623_at	0,00857	-2,35114
CLDN10	Claudin 10	205328_at	0,00861	-2,78141
TMEM150C	Transmembrane protein 150C	229623_at	0,00914	-3,16840
PRSS23	Protease, serine, 23	226279_at	0,00927	-2,09789
TMC5	Transmembrane channel-like 5	219580_s_at	0,01044	-2,58058
ALDH1A1	Aldehyde dehydrogenase 1 family, member A1	212224_at	0,01079	-2,55132
SNTN	Sentan, cilia apical structure protein	239150_at	0,01143	-3,24422
RARRES1	Retinoic acid receptor responder 1	206392_s_at	0,01180	-2,87922
CYP4B1	Cytochrome P450, family 4, subfamily B, polypeptide 1	210096_at	0,01216	-2,54099
ADH1C	Alcohol dehydrogenase 1C (class I)	206262_at	0,01333	-3,01050
GALNT12	Polypeptide GalNAc transferase 12	218885_s_at	0,01351	-1,89640
NR2F2	Nuclear receptor subfamily 2, group F, member 2	209120_at	0,01357	-2,41105
LOC100506582	n/a	238720_at	0,01383	-3,96209
PROM1	Prominin 1	204304_s_at	0,01456	-3,12999
MECOM	MDS1 and EVI1 complex locus	226420_at	0,01699	-2,34179
CRNDE	Colorectal neoplasia	238021_s_at	0,02083	-2,23300
TF	Transferrin	203400_s_at	0,02149	-2,84610
RARRES1	Retinoic acid receptor responder 1	221872_at	0,02280	-1,81951
TF	Transferrin	214063_s_at	0,02449	-3,05918
CXCL11	Chemokine ligand 11	210163_at	0,02461	-1,61067
SOX2	SRY-box 2	228038_at	0,02469	-2,51238
C11orf70	n/a	224463_s_at	0,02477	-2,25622
IGHG1, IGHG2, IGHM, IGHV4-31	Immunoglobulin heavy constant gamma 1, 2, mu and variable 4-31	211430_s_at	0,02637	-2,32966
CDC20B	Cell division cycle 20 homolog B	240161_s_at	0,05148	-2,54067

Supplemental Table 12.2 Full Gene List based on Need for Mechanical Ventilation before 2.5 log cut-off.

Gene		Probe Set ID	*p-value*	Median fold change
HLA-DQA1/DQA2	Major histocompatibility complex, class II, DQ alpha 1/2	212671_s_at	0,00000	-2,84819
TRIB2	Tribbles homolog 2 (Drosophila)	202478_at	0,00000	-2,18414
NR4A2	Nuclear receptor subfamily 4, group A, member 2	204622_x_at	0,00000	-2,02076
NR4A2	Nuclear receptor subfamily 4, group A, member 2	216248_s_at	0,00000	-1,95761
MUC13	Mucin 13	218687_s_at	0,00002	-3,40989
TNFSF10	Tumor necrosis factor superfamily, member 10	202688_at	0,00002	-2,49353
MXRA5	Matrix-remodelling associated 5	209596_at	0,00002	-2,02161
GZMA	Granzyme A	205488_at	0,00003	-2,70209
CFB	Complement factor B	202357_s_at	0,00003	-2,53650
LGALS3BP	Lectin, galactoside-binding, soluble, 3 binding protein	200923_at	0,00003	-1,91567
UBD	Ubiquitin D	205890_s_at	0,00004	-3,54059
MSP	β-microseminoprotein	210297_s_at	0,00005	-2,91614
GBP1	Guanylate binding protein 1	202270_at	0,00007	-1,78777
AKR1C1	Aldo-keto reductase family 1, member C1	216594_x_at	0,00009	-2,49039
CLEC5A	C-type lectin domain family 5, member A	219890_at	0,00010	2,36898
AKR1C2	Aldo-keto reductase family 1, member C2	209699_x_at	0,00011	-2,89072
AKR1C1	Aldo-keto reductase family 1, member C1	204151_x_at	0,00011	-2,61119
SPINK1	Serine peptidase inhibitor, Kazal type 1	206239_s_at	0,00011	2,32174
MSP	β-microseminoprotein	207430_s_at	0,00013	-2,73207
GZMB	Granzyme B	210164_at	0,00015	-2,70688
AQP3	Aquaporin 3	39248_at	0,00018	-2,95154
TFF1	Trefoil factor 1	205009_at	0,00019	-2,53729
GNLY	Granulysin	37145_at	0,00022	-1,93698
Unannotated	n/a	235229_at	0,00023	-2,20729
SYTL2	Synaptotagmin-like 2	232914_s_at	0,00026	-2,04847
UPK1B	Uroplakin 1B	210065_s_at	0,00030	-2,31083
SAA1, SAA2	Serum amyloid A1, serum amyloid A2	214456_x_at	0,00035	-2,34207

Supplemental Table 12.2 Continued.

Gene		Probe Set ID	*p-value*	Median fold change
ALDH1A1	Aldehyde dehydrogenase 1 family, member A1	212224_at	0,00043	-2,41902
NCOA7	Nuclear receptor coactivator 7	225344_at	0,00046	-2,08015
CCL7	Chemokine ligand 7	208075_s_at	0,00061	2,70079
HMGN3	High mobility group nucleosomal binding domain 3	209377_s_at	0,00068	-1,79715
TOX3	TOX high mobility group box family member 3	214774_x_at	0,00072	-2,19080
GALNT12	Polypeptide GalNAc transferase 12	218885_s_at	0,00072	-1,79081
WFDC2	WAP four-disulfide core domain 2	203892_at	0,00073	-1,94166

Supplemental Table 12.3 Gene List based on Need for Supportive Care.

Gene		Probe Set ID	*p-value*	Median fold change
MSP	β-microseminoprotein	210297_s_at	0.00158	-3.84
SYTL2	Synaptotagmin-like 2	232914_s_at	0.00177	-2.67
MSP	β-microseminoprotein	207430_s_at	0.00178	-4.34
UBD	Ubiquitin D	205890_s_at	0.00271	-3.50
TSPAN8	Tetraspanin 8	203824_at	0.00429	-3.39
IGLV1-44	Immunoglobulin lambda variable 1-44	214677_x_at	0.00464	-3.17
Unannotated	n/a	235229_at	0.00521	-2.88
Unannotated	n/a	238103_at	0.00559	-2.86
CP	Ceruloplasmin	227253_at	0.00568	-3.00
C9orf135	n/a	243610_at	0.00582	-3.47
MUC13	Mucin 13	218687_s_at	0.00593	-3.54
TMC5	Transmembrane channel-like 5	240304_s_at	0.00653	-2.70
SCGB1A1	Uteroglobin	205725_at	0.00792	-2.95
CLDN10	Claudin 10	205328_at	0.00861	-2.78
TMEM150C	Transmembrane protein 150C	229623_at	0.00914	-3.17

Supplemental Table 12.4 Genes List based on Need for Mechanical Ventilation.

Gene		Probe Set ID	*p-value*	Median fold change
HLA-DQA1/DQA2	Major histocompatibility complex, class II, DQ alpha 1/2	212671_s_at	0.00000	-2.85
MUC13	Mucin 13, cell surface associated	218687_s_at	0.00002	-3.41
CFB	Complement factor B	202357_s_at	0.00003	-2.54
GZMA	Granzyme A	205488_at	0.00003	-2.70
UBD	Ubiquitin D	205890_s_at	0.00004	-3.54
MSP	microseminoprotein, beta-	210297_s_at	0.00005	-2.92
AKR1C2	Dihydrodiol dehydrogenase 2	209699_x_at	0.00011	-2.89
AKR1C1	Dihydrodiol dehydrogenase 1	204151_x_at	0.00011	-2.61
MSP	βmicroseminoprotein	207430_s_at	0.00013	-2.73
GZMB	Granzyme B	210164_at	0.00015	-2.71
AQP3	Aquaporin 3	39248_at	0.00018	-2.95
TFF1	Trefoil factor 1	205009_at	0.00019	-2.54
CCL7	Chemokine ligand 7	208075_s_at	0.00061	2.70
WDR78	WD repeat domain 78	1554140_at	0.00082	-2.56
LOC100506582	n/a	238720_at	0.00104	-3.28
TSPAN8	Tetraspanin 8	203824_at	0.00157	-2.81
C20orf114	n/a	226067_at	0.00184	-2.82

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High pneumococcal density correlates with more mucosal inflammation and reduced respiratory syncytial virus disease severity in infants

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Abstract

Respiratory syncytial virus (RSV) is an important cause of lower respiratory tract infections in infants. A small percentage of the infected infants develops a severe infection, while most of these severely ill patients were previously healthy. It remains unclear why these children develop severe RSV infections. In this study, we investigate whether pneumococcal nasopharyngeal carriage patterns correlate with mucosal inflammation and severity of disease.

In total, 105 infants hospitalized with RSV infection were included and recovery samples were taken from 42 patients. The presence and density of *Streptococcus pneumoniae* was determined by RT qPCR to study its relation to viral load, inflammation (MMP-9 and IL-6) and severity of RSV disease.

We show that pneumococcal presence or absence in the nasopharynx does not correlate with viral load, inflammation or severity of disease. However, when pneumococcus is present in patients, a higher nasopharyngeal pneumococcal density was correlated with a higher RSV load, higher MMP-9 levels and a less severe course of disease.

Our results show correlations between *S. pneumoniae* density and viral load, inflammation and disease severity, suggesting that pneumococcal density may be an indicator for severity in paediatric RSV disease.

Background

Respiratory syncytial virus (RSV) is a major cause of severe respiratory infections in infants below 6 months of age and the most common cause for bronchiolitis. Approximately 60% of all infants are infected with RSV during their first winter season and at the age of 2 almost all children have encountered RSV[1]. The vast majority of children will develop relatively mild symptoms, comparable to a common cold. However, approximately 2-3% will develop bronchiolitis and will be hospitalized[2]. Known risk factors for severe disease are age (<6 months), prematurity, congenital heart or lung disease and presence of siblings[2-5]. A considerable part of the severely ill patients were previously healthy and, at this moment, we do not understand why these children become severely ill.

Mucosal surfaces of the human body are inhabited by complex microbial ecosystems, together called the 'microbiome'. A growing body of evidence shows that the microbiome is crucial for the shaping of our immune system[6,7]. Because an over-exuberant immune response plays a crucial role in severe RSV infections, the composition of the microbiome during the first months after birth should be considered as a potential determinant for severity of disease upon infection with RSV[8]. Interactions between RSV and *Streptococcus pneumoniae* are well-documented previously. Most of these studies focus on the influence of RSV infections on secondary pneumococcal infections, e.g. showing an enhanced adherence of *S. pneumoniae* to RSV-infected cells[9-13]. However, whether the presence of *S. pneumoniae* in the nasopharynx may influence a subsequent RSV infection has not been studied in infants. There are studies showing that *S. pneumoniae* may aggravate RSV infections[14,15]. Cells infected by pneumococci are more susceptible to RSV infection in vitro and in a mouse model[14]. In a study in South-Africa, it was shown that vaccination against *S. pneumoniae* reduces viral-caused pneumonias by 31%, suggesting a promoting role for *S. pneumoniae* in viral respiratory infections[15]. In this study, the presence and density of *S. pneumoniae* was determined in a 66 clinical cohort of infants hospitalized with RSV infections. Classically, severity of an infection is thought to be dependent on two factors: pathogen load and inflammatory response. Previous studies have shown that bacterial colonization is able to influence viral infection rate[16-18], but may also influence the inflammatory response during an infection[19-22]. Therefore, we studied correlations between pneumococcal colonization patterns and RSV load, levels of the inflammatory mediators IL-6 and MMP-9, both associated with RSV infection[23-25] as well as *S. pneumoniae* infection[26-28], and severity of disease.

Methods

Study design

Children younger than 2 years of age with laboratory confirmed viral respiratory tract infections were prospectively included during three consecutive winter seasons

(November-April in 2010/2011, 2011/2012 and 2012/2013). Written informed consent was obtained from all parents. Patients with congenital heart or lung disease, immunodeficiency or glucocorticoid use were excluded. Medical history, demographics and clinical parameters were collected from questionnaires and medical records. Patients were divided into three groups. Children without hypoxia were classified as 'mildly ill'. 'Moderately ill' children received supplemental oxygen, while 'severely ill' children required mechanical ventilation. Within 24h after admission, a nasopharyngeal aspirate (NPA) was collected (acute) and parents from hospitalized children were asked for permission to draw a second NPA sample 4-6 weeks after admission (recovery). The study was approved by the Central Committee on Research Involving Human Subjects of the Radboud university medical center.

Sample collection

The nasopharyngeal aspirates were collected by introducing a catheter, connected to a collection tube and an aspiration system, into the nasopharyngeal cavity. Then, 0.5 ml of saline was instilled into the catheter and, while slowly retracting the catheter, the nasopharyngeal fluid was aspirated in a collection tube. Afterwards the catheter was flushed with 1 ml of saline and this was added to the collection fluid. Samples were kept cold and were immediately transferred to the laboratory. Samples were taken for viral and bacterial diagnostics. For viral diagnostics samples were analyzed by multiplex PCR, quantifying 15 different viral pathogens, as previously described[29]. The remaining NPA was centrifuged at 500*g for 10 min at 4°C to spin down the mucus and 100 cells, after which the supernatant was frozen at -80°C for ELISA.

Bacterial diagnostics

Nasopharyngeal aspirates (300 µl) were resuspended in 343 µl lysis buffer (AGOWA magMini DNA Isolation Kit, AGOWA) with 57 µl protease. Then, 25-50 mg sterile zirconium beads were added and 500 µl phenol. The samples were disrupted using the TissueLyser (Qiagen) for 2 min, twice. The samples were then centrifuged for 10 min at 10,000 rpm and the supernatant containing the released DNA was then purified according to the protocol included in the AGOWA mag Mini DNA Isolation Kit, as described previously[30]. Samples were resuspended in 50 µl elution buffer and stored at -80°C until further use. RT qPCR was used to quantify total bacterial carriage density (16s), *S. pneumoniae* (Sp), and *H. influenza* (Hi) by amplifying the 16s rRNA gene, the lytA gene and the hpd gene, respectively, as previously described[30]. Primers and probes used can be found in supplemental table 1. All samples were run in duplicate. Samples were analyzed on a Bio-Rad CFX96 Real-Time System. Primer and probe concentrations were optimized for each target and the machine. Final primer/probe concentrations were 5 nM for 16s, 200 nM for lytA (Sp) and 300 nM for hpd (Hi). 0.8 µl of each primer and/or probe was added to a 20 µl reaction volume. Standard curves were created using purified genomic DNA extracted from laboratory reference strains and quantified using the NanoDrop ND-1000. For *S. pneumoniae*, DNA was extracted

from TIGR4[31]. For *H. influenzae*, DNA was extracted from R2866[32]. As a measure for bacterial density, we determined the number of bacterial genomes per ml of NPA. We used the following formula: Number of genome copies per μl of extracted DNA = (mass in ng * Avagadro's number) / (genome length for each bacteria * 109 * 650). 650 daltons is the average weight of a DNA basepair. We then multiplied this number by 167 to account for the difference between the volume of nasal wash used 125 in the extraction (300 μl) and the volume of extracted DNA (50 μl) in order to determine genome copies per ml of NPA[30]. As a negative control, a water sample was included during the whole procedure from DNA isolation to RT qPCR. For the specific bacterial RT qPCR's Ct values above 35 were regarded as negative, based on the negative controls. For the 16s RT qPCR, Ct values above 30 were regarded as negative.

Inflammatory markers

MMP-9 concentrations were measured in the nasopharyngeal aspirates using R&D ELISA kits (R&D systems) according to the instructions of the manufacturer. Samples were 10,000x diluted for the MMP-9 ELISA, which therefore had a lower detection limit of 156 ng/ml. IL-6 concentrations were measured using a Sanquin ELISA kit according to the instructions of the manufacturer. Samples were 100-fold diluted and therefore the IL-6 ELISA had a lower detection limit of 156 pg/ml.

Statistical analysis

Values are expressed as percentages for categorical variables and as median and interquartile range (IQR) for continuous variables. Chi-squared tests were performed to compare categorical data between multiple groups. When significant differences were identified, Fisher's exact tests were performed to specify which groups differed. As tested by Shapiro-Wilk's test, none of the continuous variables were normally distributed. Therefore, Kruskal-Wallis H tests were used to compare continuous data between multiple groups. When significant differences were found, Mann-Whitney U tests were performed to specify which groups differed. To determine whether correlations existed, a Spearman correlation test was performed. A value of $p<0.05$ was considered statistically significant. All statistical analyses were conducted in GraphPad Prism 5.03 or IBM SPSS Statistics 20.

Results

Patient characteristics

In total, 105 RSV-infected infants were included. From these 105 RSV infected patients, 25 patients were categorized as mildly ill meaning there was no need for oxygen support, 53 patients were moderately ill and required oxygen support, 27 patients were severely ill necessitating mechanical ventilation. Significant differences between the severity groups were found for known risk factors like age and presence of siblings, but

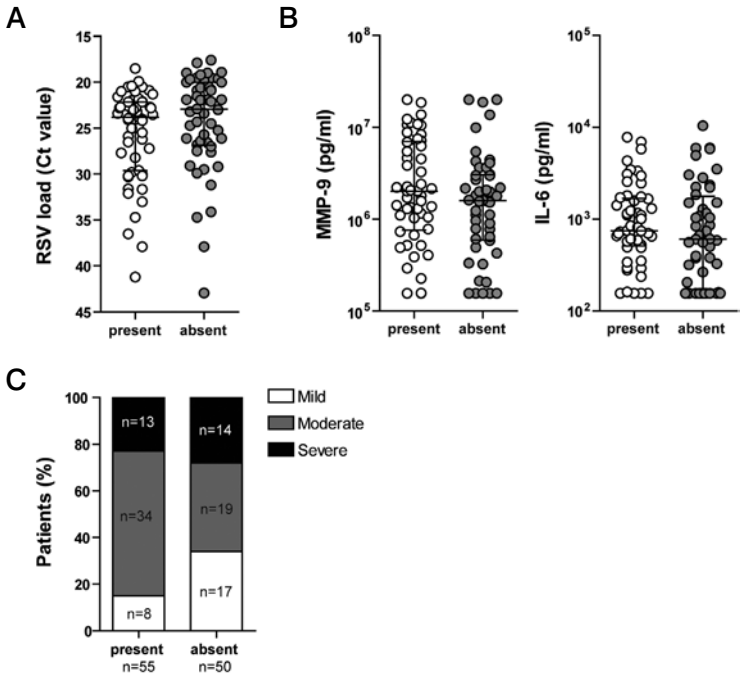

Figure 13.1 Pneumococcal presence does not correlate with RSV load, inflammation or disease severity. Viral load (**A**) and MMP-9 and IL-6 (**B**) were compared between the group positive for S. pneumoniae and the group negative for S. pneumoniae. Data shown are median ± IQR. Data were tested for significant differences using a Mann-Whitney U test. Pneumococcal presence was compared between the three severity groups (**C**). Differences in bacterial colonization rates were compared using Chi-square tests. When significant differences were found, Fisher's exact tests were performed to specify which groups differed significantly. The moderately ill infants had a significantly higher colonization rate compared to the mildly infected infants (***$p < 0.001$).

also for hospital duration, daycare attendance, vaccination status and viral co-infections (**table 13.1**).

Presence of *S. pneumoniae* does not correlate with viral load, inflammation or disease severity

In all collected samples, 16s rRNA could be detected by RT qPCR. Samples could therefore be used for additional analysis (data not shown). First, we studied whether pneumococcal colonization influenced RSV infection. There were no significant differences between the pneumococcal positive group compared to the pneumococcal negative group, regarding viral load (**figure 13.1A**). This was also the case for the inflammatory mediators, MMP-9 and IL-6 (**figure 13.1B**). When looking at severity

scores, the moderately ill infants had a higher percentage of pneumococcal positive infants compared to the mildly ill infants (**figure 13.1C**). However, we did not see this trend for the severe group.
In conclusion, we detected no correlation between the presence of *S. pneumoniae* and viral load, inflammation or severity of disease. As shown in **table 13.1**, age is an important potential confounder. Therefore, we checked whether pneumococcal colonization was correlated with age (**supplemental figure 13.1A**). Some significant differences were found between the different age groups but no clear trends were found. The same analyses were performed for another potentially pathogenic nasopharyngeal bacterium (*Haemophilus influenzae*) to see if the effects found were specific for *S. pneumoniae*. No significant differences regarding viral load, inflammation and severity were found for *H. influenzae* (**supplemental figure 13.2**). Colonization with *H. influenzae* was also not age-dependent (**supplemental figure 13.1B**).

Pneumoccocal density correlates with viral load, inflammation and severity

As we did not see any differences between patients with or without pneumococcus, we then focussed on the patients that were pneumococcal positive. We studied whether pneumococcal density influences RSV infection. When correlating pneumococcal density with RSV load, we found that a higher density of *S. pneumoniae* was correlated with higher titers of RSV (**figure 13.2A)**. This also holds true for MMP-9, for which a higher *S. pneumoniae* density correlated with higher concentrations of MMP-9. In contrast, IL-6 did not correlate with pneumococcal density (**figure 13.2B)**. Lastly, we also compared the different severity groups and found that more severely infected infants had lower pneumococcal densities (**figure 13.2C)**.
In addition, pneumococcal density was not significantly different between the different age groups (**supplemental figure 13.3A**) and therefore not age-dependent.
H. influenzae was also included, but no significant differences were found (**supplemental figure 13.4**) and *H. influenzae* density was not age-dependent (**supplemental figure 13.3B**). These results indicate that the effects found are specific for *S. pneumoniae*.

Pneumococcal colonization and density does not change after RSV infection

Additionally, 42 of the patients were also sampled 4-6 weeks after hospital discharge. Because viral respiratory infections are known to increase the risk of bacterial infections, we investigated whether pneumococcal colonization rates and density change after an RSV infection. Percentages of colonization were identical when comparing the acute and recovery group (**figure 13.3A**). Therefore, RSV infection did not change colonization rates in infants. Also, pneumococcal density of the pneumococcal positive patients was not significantly different between patients having an infection and patients in the recovery phase (**figure 13.3B**). Lastly, when we looked at the shifts in colonization, we saw that 25% of the infants was not colonized during the acute infection and remained

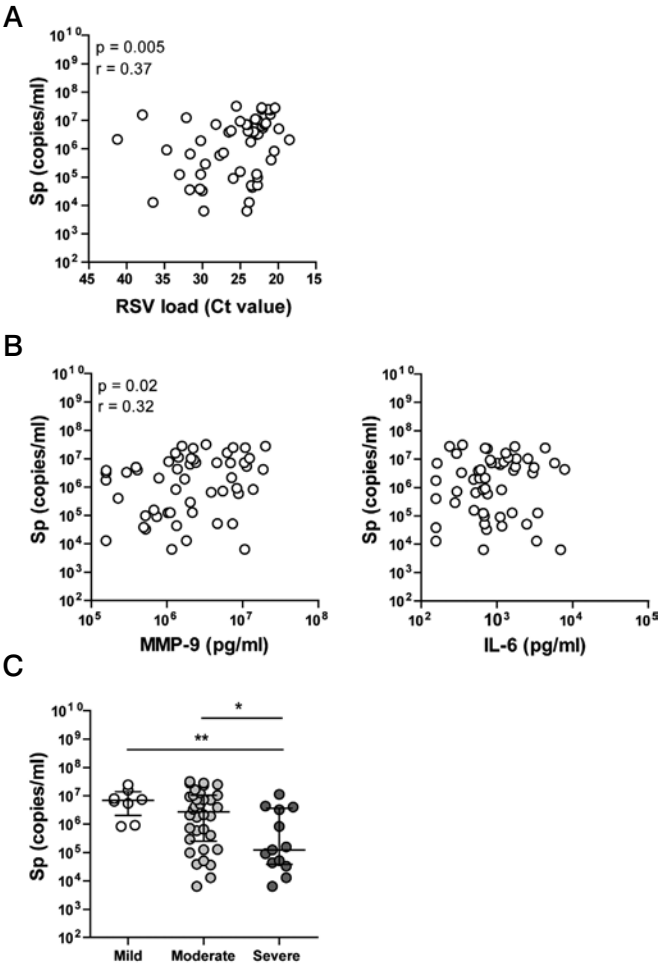

Figure 13.2 Pneumococcal density correlates with RSV load, inflammation and severity. Pneumococcal density was correlated with viral load (**A**) and MMP-9 and IL-6 levels (**B**). Correlations were tested for significance using a Spearman correlation test. Pneumococcal density was compared between the three severity groups (**C**). Data shown are median ± IQR. Differences in bacterial carriage density are tested using a Kruskal-Wallis test. When significant differences were found, Mann-Whitney U tests were performed to specify which groups differed (*$p < 0.05$, **$p < 0.01$).

so during the recovery phase (**figure 13.3C**). Also, approximately the same number of infants acquired *S. pneumoniae* after an RSV infection as the number losing *S. pneumoniae*.

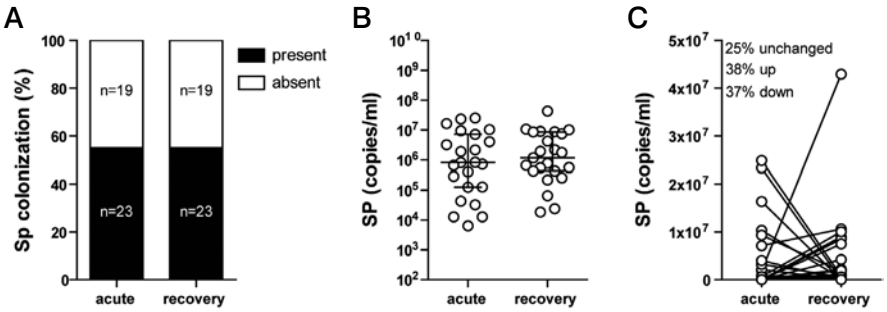

Figure 13.3 Pneumococcal colonization and density does not change after RSV infection. Pneumococcal colonization rate, density and shifts were compared between the acute and the recovery group. Differences in bacterial colonization rates were compared using a Fisher's exact test of all patients that had an acute and recovery sample taken (**A**). Differences in bacterial density between de pneumococcal positive samples were compared using a Mann-Whitney U test. Data shown are median ± IQR (**B**). Shifts in pneumococcal density between all acute and recovery samples are shown (**C**).

Discussion

This study is the first to report that *S. pneumoniae* density in the nasopharynx is correlated with viral load, inflammatory conditions and severity during RSV infection of hospitalized infants. We here show that pneumococcal carriage density is lower during severe RSV infections and that a low density correlates with a low RSV load and low levels of MMP-9. This indicates that *S. pneumoniae* colonization during RSV infection is associated with viral infection and the inflammatory response and therefore could influence the development of disease.

We used RT qPCR to determine bacterial carriage density instead of culturing methods. RT qPCR is faster and more sensitive than culturing techniques, especially when samples have to be frozen[33, 34]. Good correlations have been reported for *S. pneumoniae* and *H. influenzae* when comparing RT qPCR with quantitative culturing[35]. One important drawback of RT qPCR is that viable and nonviable bacteria cannot be distinguished. However, for our study, this is an advantage as it more accurately detects the presence of bacterial pathogens without any disturbance due to recent antibiotic treatment. In the Netherlands, patients with severe infections, who require mechanical ventilation, receive selective decontamination of the digestive tract (SDD) by use of antibiotics. Our study subjects were sampled within 24h after admission to the hospital. Therefore, we do not expect that the SDD regimen will have exerted an effect on bacterial carriage density measured by RT qPCR. Moreover, if SDD antibiotics would have such rapid effects, one would also expect to see the same trends for *H. influenzae*, but this is not the case.

A recent study has shown that antibiotics given in the week prior to sampling reduced colonization rates of potential pathogenic bacteria, e.g. *S. pneumoniae*, *M. catarrhalis*, *H. influenzae*, *S. aureus* and β*hemolytic Streptococcus* but had no clear effect on paediatric RSV disease severity scores[36]. In our study, we determined whether infants received antibiotic treatment in the 4 weeks prior to hospitalization. No significant differences were found in pneumococcal colonization or severity status (data not shown).

We included a control group of infants who were hospitalized for a hernia operation to check how the inflammatory mediators behave in different groups. We see different dynamics for MMP-9 and IL-6 (supplemental figure 5). IL-6 is only elevated during the acute phase of disease, whereas MMP-9 is elevated both during the acute phase of disease and the recovery phase.

To our knowledge, we are the first to show a negative correlation between RSV disease severity and pneumococcal carriage density. Clinical studies have shown that the pneumococcal conjugate vaccine also results in a reduction of 31% of the viral respiratory tract infections[15,38]. This suggests that colonization with *S. pneumoniae* increases the risk of viral infection or that its presence enhances symptoms. However, our study shows that severely ill patients have lower pneumococcal carriage density, thus suggesting that *high S. pneumoniae* density protects against severe infections.

S. pneumoniae and RSV are known to influence each other[37]. In vitro studies have shown that RSV infection of respiratory epithelial cells enhances the adherence of *S. pneumoniae*, possibly because the RSV G protein can serve as a receptor for *S. pneumoniae*[9, 12]. This is in accordance with our observation that higher RSV loads coincide with higher pneumococcal carriage density.

MMP-9 is a matrix metalloproteinase which is involved in breakdown of extracellular matrix and appears to be a regulatory factor in neutrophil migration across the basement membrane[39]. High MMP-9 concentrations have been associated with severe RSV infections[24]. Studies have shown that RSV is able to induce MMP-9 production[40]. However, in our study no correlation was found between MMP-9 levels and RSV load (data not shown). Previously, we have shown that *S. pneumoniae* is able to induce high MMP-9 levels under in vitro conditions[26]. This is supported by this study where we show a correlation between pneumococcal carriage density and MMP-9 levels, in which higher numbers of *S. pneumoniae* led to higher concentrations of MMP-9.

All our data together suggest that severely ill infants have lower pneumococcal loads, these lower pneumococcal loads are correlated with lower RSV levels and lower MMP-9 levels. However, this suggests indirectly that severely ill infants have lower RSV levels, which seems contradictory. Although the general consensus is that viral load is probably correlated with disease severity, there is still discussion to what extent[41-44]. Our data do not show a correlation between RSV load and disease severity (Table I). A possible explanation for this discrepancy in our results is that at the moment the infants are included, they are in an advanced stage of disease. It is therefore possible that in the severe cases the virus has already partly been cleared and that severe inflammation is

cause of the severity, not viral load. Another explanation is that the interactions between *S. pneumoniae*, RSV load, inflammation and disease severity are multidirectional and more complex than we can grasp in this study. Other factors, that we did not include in this study, could play a role. We did carefully evaluated the presence of potential confounders that may explain the results of our study. When comparing severity groups, the severe groups had a lower daycare attendance and lower vaccination rates. These are both due to the fact that the severely ill patients were often too young for vaccination and daycare attendance. The severely infected patients also had more siblings, which is a known risk factor for severe RSV infections[4,46]. Vaccination, daycare attendance and presence of siblings can all influence pneumococcal carriage patterns. However, the severely infected infants did not yet receive pneumococcal vaccination and had more siblings. This would all have resulted in a higher pneumococcal load instead of a lower load. We cannot exclude that less daycare attendance in the severely infected infants may have contributed to lower pneumococcal carriage. Patients with severe infections had more RSV mono-infections compared to the other severity groups. This was already shown by our group in a previous cohort[3]. Finally, age could be a confounder as the severely ill patients are significantly younger compared to the moderate or mildly ill patients. As we have shown in supplemental figure 1 and 3, *S. pneumoniae* and *H. influenzae* carriage densities were not dependent on age, whereas the densities of *S. aureus* and *M. catarrhalis* were age dependent (data not shown).

There are some limitations to our study. Based on the study design we cannot state anything on causality. We do not know whether pneumococcal density changes as a result of RSV infection, or whether the pneumococcal density was already different and potentially influenced susceptibility to an RSV infection. A prospective study is needed to definitely determine whether *S. pneumoniae* influences RSV severity. Moreover, there is a difference between absolute density and relative abundance. We did not look at the influence of relative abundance of *S. pneumoniae*.

Conclusions

In summary, we here show that *S. pneumoniae* density correlates to disease severity, viral load and inflammatory mediators, which suggests that *S. pneumoniae* density influences both viral load as well as the mucosal inflammatory response during an RSV infection. Once we understand the role of different bacteria residing in the upper respiratory tract in severity of RSV infections, we might be able to predict severity or modify the composition of the microbiome to prevent severe infections.

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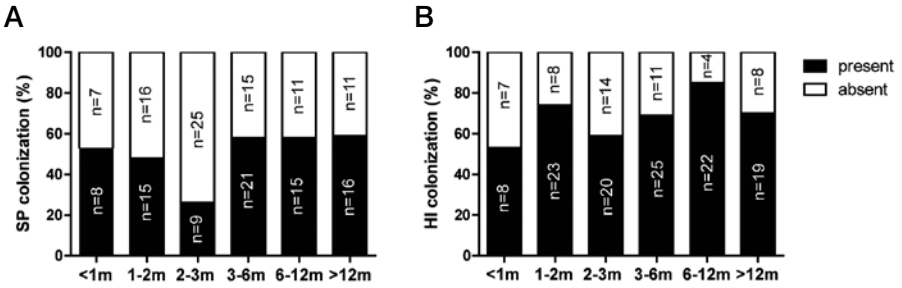
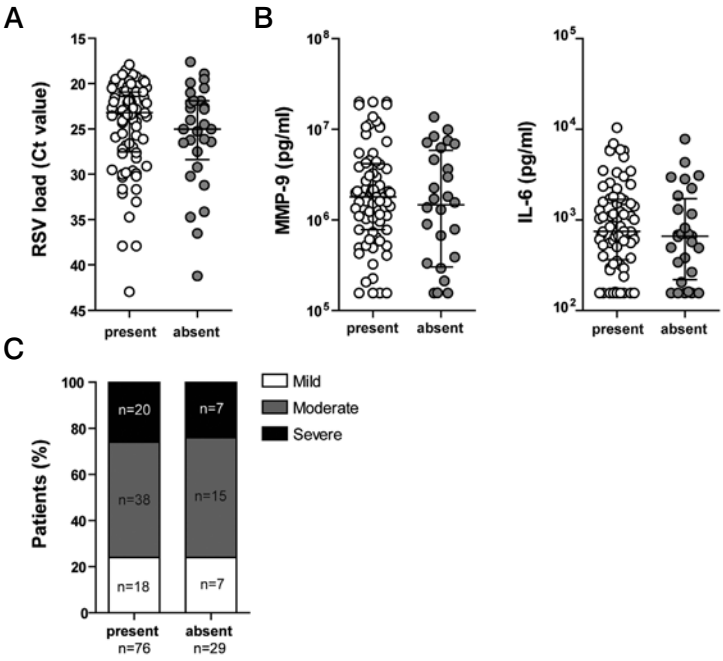
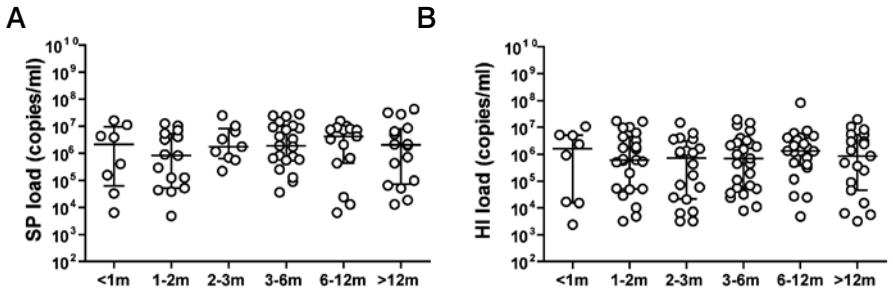


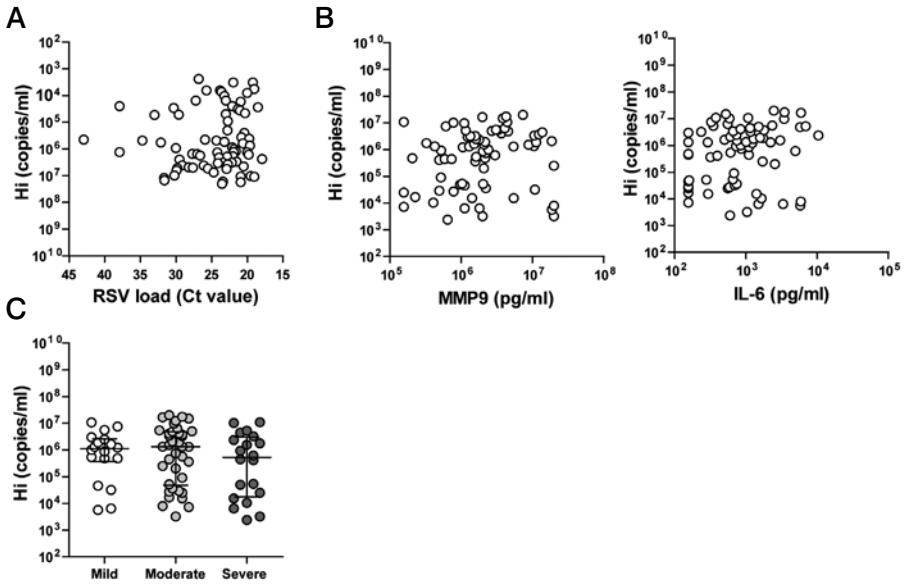
Supplemental Figure 13.1 No clear age dependent colonization patterns were found for *S. pneumoniae* (**A**) and *H. influenza* (**B**). Differences in bacterial colonization rates were compared using Chisquare tests. When significant differences were found, Fisher's exact tests were performed to specify which groups differed. Significant differences were found but no clear trend was visible.



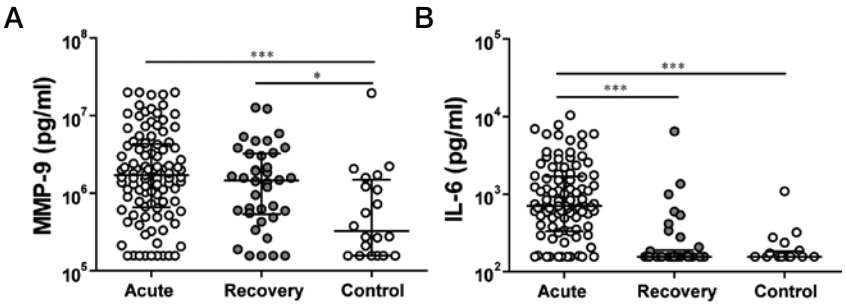
Supplemental Figure 13.2 *H. influenzae* presence does not influence RSV load, inflammation or severity. Viral load (**A**) and MMP-9 and IL-6 (**B**) were compared between the group positive for *H. influenzae* and the group negative for *H. influenzae*. Data shown are median ± IQR. Data were tested for significant differences using a Mann-Whitney U test. *H. influenzae* presence was compared between the three severity groups (**C**). Differences in bacterial colonization rates were compared using Chi- square tests. No significant differences were found.



Supplemental Figure 13.3 *S. pneumoniae* (**A**) and *H. influenza* (**B**) density are not age dependent. Data shown are median ± IQR. Differences in bacterial densities are tested using the Kruskal-Wallis test. No significant differences were found.



Supplemental Figure 13.4 *H. influenzae* density does not influence RSV load, inflammation and severity. *H. influenzae* density was correlated with viral load (**A**) and MMP-9 and IL-6 levels (**B**). Correlations were tested for significance using a Spearman correlation test. *H. influenzae* carriage density was compared between the three severity groups (**C**). Data shown are median ± IQR. Differences in bacterial carriage density are tested using the Kruskal-Wallis test. No significant differences were found.



Supplemental Figure 13.5 IL-6 levels are elevated during acute phase of disease, whereas MMP-9 is elevated during acute and recovery phase of disease. MMP-9 levels **(A)** and IL-6 levels **(B)** were measured during the acute and recovery phase of disease and were compared to a control group of healthy patients. Data shown are median ± IQR. Differences in cytokine levels are tested using the Kruskal-Wallis test. When significant differences were found the different groups were compared using a Mann-Whitney U test (*$p<0.05$, ***$p<0.001$).

14

Mucosal IgG levels correlate better with respiratory syncytial virus load and inflammation than plasma IgG levels

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Abstract

Maternal vaccination is currently considered as a strategy against RSV infections. In RSV infected infants, high mucosal IgG levels correlated better with reduced RSV load and lower mucosal CXCL10 levels than plasma IgG levels. For future vaccination strategies against RSV, more focus should be on the mucosal humoral immune response.

Introduction

The first description of respiratory syncytial virus (RSV) was 60 years ago[1, 2]. Despite the long awareness of the virus and the morbidity it causes, still no vaccines are available. This may partly be due to one of the first vaccination trials in the sixties, which had a devastating effect on RSV vaccine development. But there are more hurdles to be taken in RSV vaccine development. An important difficulty is the fact that the largest target group are very young infants (<6 months of age), who may respond inadequately to vaccination. Also, RSV is very efficient in evading the immune response as is shown by reinfections throughout live and lastly, there is no animal model that is fully permissive to human RSV infection.

Most vaccines aim to induce pathogen-specific IgGs. Palivizumab, a passively administered neutralizing monoclonal antibody, is able to protect infants from severe RSV disease[3-5]. This shows antibodies are able to prevent severe RSV infections and induction of neutralizing antibodies by vaccination could potentially work. A vaccination route that is often considered for RSV and which resembles passive immunization, is maternal vaccination. High levels of maternally-derived RSV specific antibody, measured in the sera of infants, protects against RSV infection during the first months of life[6-8]. Maternal vaccination aims to enhance the maternally derived IgG antibody levels in the infant.

However, it is unknown if plasma IgGs also reach the mucosal locations, if plasma and mucosal IgG levels are correlated with each other and if both are equally protective. To answer these questions, we studied maternally derived pre-existing RSV-specific plasma and mucosal antibody titers and their correlation with RSV load and RSV-associated inflammation, i.e. CXCL10, in a clinical paediatric cohort.

23 hospitalized children less than 3 months of age with laboratory confirmed RSV infections were prospectively included during two consecutive winter seasons (November-April in 2010-2011 and 2011-2012). Patients with congenital heart or lung disease, immunodeficiency or glucocorticoid use were excluded. Written informed consent was obtained from all parents of patients. The study was approved by the Central Committee on Research involving Human Subjects of the Radboudumc. Demographics and clinical parameters were collected from questionnaires and medical records. Within 24h after admission, a blood sample and a nasopharyngeal aspirate (NPA) were collected as previously described[9].

The mean age of the patients was 53 days, the average gestational age 38 weeks and 48% of the patients was male (**table 14.1**). Regarding their disease status, mean duration of hospitalization was almost 10 days and the average RSV load gave a Ct value of 25. The young age of the infants enhances the chance that this was their primary RSV infection and therefore only maternal antibodies are studied. Moreover, from literature it is known that infants do not mount significant neutralising antibody responses before the age of 4 months[10].

Table 14.1 Patient characteristics

	RSV infected patients (n=23)
Age [median age in days ± IQR]	53 [31-70]
Mae (%)	11 [48]
Gestational age [median age in weeks ± IQR]	38.4 [38.3 - 38.5]
RSV load [median load n Ct value ± IQR]	24.8 [24.6 – 25.0]
Duration of hospitalization [median duration in days ± IQR]	9.6 [9.4 – 9.7]

Maternally derived RSV-specific IgGs were measured in both plasma and nasal aspirates of patients by ELISA. 96-wells plates (Nunc Maxisorp) were coated with 1:200 diluted whole RSV-A2 ($4x10^7$ FITC-detected infectious particles/ml) in PBS (Lonza). RSV-A2 was cultured and quantified as described previously[11]. Plates were incubated for 5 hours at 4°C, washed (PBS with 0.05% Tween-20) and blocked for at least 2 hours with 100μl PBS with 1% BSA (Sigma-Aldrich). A standard for IgG determination was prepared using two healthy volunteers. Samples were 1:10 diluted in duplo and incubated for 2 hours at room temperature. After washing, AP-conjugated antibody against human IgG (1:10,000 in 1%BSA) (Southern Biotech) was added and incubated for 2 hours at room temperature. After washing, substrate buffer (10 mM diethanolamine with 0.5mM $MgCl_2$) was added. Absorbance was measured at 450 nm and 690 nm after 30 minutes and 60 minutes. Background binding was substracted from each sample measurement and results were calculated to arbitrary units (AU). In our pediatric cohort, no correlation was found between the levels of mucosal and plasma IgGs (**figure 14.1A**), suggesting that, next to passive transudation, additional mechanisms are at play during infection. It has been shown that, due to an infection, IgG antibodies can be actively secreted to the lumen[12].

For viral diagnostics, samples were analyzed by multiplex PCR, quantifying 15 different viral pathogens, as previously described[13]. In contrast to plasma IgG levels (**figure 14.1B**), mucosal IgG levels were correlated with RSV load in which higher mucosal IgG levels resulted in a lower RSV load (**figure 14.1B**). This shows that mucosal IgG levels are a better correlate for viral load.

High CXCL10 plasma levels are indicative for RSV-associated inflammation[14]. Therefore, we tested whether mucosal and plasma CXCL10 levels correlated with RSV load. The concentration of CXCL10 was determined using a Cytometric Bead Array (CBA), according to the manufacturer's protocol (BD Biosciences). Briefly, CXCL10 levels of individual plasma samples (50μl) were analyzed, in duplicate, using the CBA kit on an LSR II flow cytometer. We found that a higher viral load resulted in higher mucosal CXCL10 levels, but not in higher plasma CXCL10 levels (**figure 14.1C**). Therefore, mucosal CXCL10 is a better correlate for viral load than plasma CXCL10.

Finally, mucosal CXCL10 levels were correlated with plasma and mucosal IgG levels. We found a significant correlation showing higher mucosal IgG levels resulted in lower

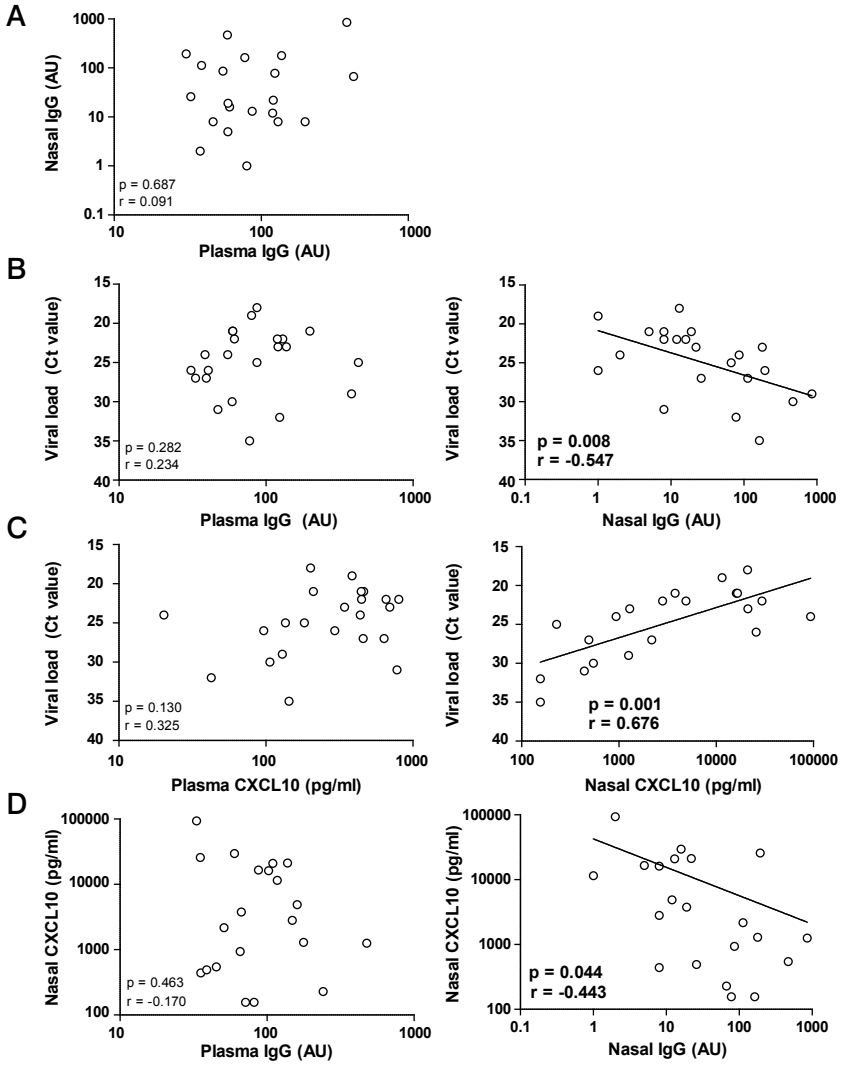

Figure 14.1 Mucosal IgG levels correlate better with respiratory syncytial virus load than plasma IgG levels. Mucosal and plasma samples were taken from RSV-infected infants. Viral load, mucosal and plasma IgG levels and mucosal and plasma CXCL10 levels were determined. Correlation analyses were performed using a Pearson correlation test. Graph-Pad Prism 5.03 was used for statistics (GraphPad Software). Values of $p < 0.05$ were considered statistically significant. Mucosal and plasma IgG levels were not correlated (**A**). Viral load was negatively correlated with mucosal IgG levels, but not plasma IgG levels (**B**). Viral load is positively correlated with CXCL10 levels in the nose, but not in plasma (**C**). Finally, mucosal CXCL10 levels were negatively correlated with mucosal IgG levels but not plasma IgG levels (**D**).

mucosal CXCL10 levels (**figure 14.1D**). No correlation was found between mucosal CXCL10 and plasma IgG levels. This suggests that mucosal antibodies also reduce RSV-associated inflammation. As a control for confounders, none of the measured parameters were correlated with age or gender of the infants (data not shown).

IgA is the predominant immunoglobulin present in the mucosa, therefore not many studies have focused on the presence and function of IgG at this location. However, as maternal vaccination aims to enhance mainly the IgG levels of the infant, it is of importance to study whether maternally derived IgGs are present on the nasopharyngeal mucosa of the infant and whether that correlates with viral load and the immune response. Our data suggest that high levels of IgG on the nasal mucosa are able to protect against RSV infections.

Although plasma IgG levels are often used as a readout for vaccine development, for future vaccine development it should be taken into account that mucosal IgG levels are potentially of greater importance than plasma IgG levels. These results suggest that mucosal (intranasal) vaccination, which aims to evoke a strong mucosal immune response[15], may be a more effective vaccination strategy. For future studies, a group with very mild RSV infection should be included to determine what level of mucosal IgGs may protect against severe infection. Moreover, correlating maternal plasma IgG levels with mucosal IgG levels of the infant would give insight into the potential of maternal vaccination. Also, more knowledge has to be generated as to how IgG molecules are transported to the nasopharynx and whether this can be enhanced. This could lead to novel immunization strategies to improve mucosal protection by long lasting higher IgG levels in the nasopharynx.

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15

Summarizing discussion and future perspectives

Introduction

Acute lower respiratory tract infections (LRTI's) are the leading cause of childhood morbidity and mortality of children between two months and five years of age. Mortality due to LRTI is rare in the western world, but still substantial in parts of Africa, Asia and South-America. Viruses were previously considered to be less important as compared to bacteria as causative agents of (severe) LRTI. However, nowadays viruses are increasingly recognised as major pathogens in LRT in adults and in children. More than 90% of children with bronchiolitis, over 50% of children with pneumonia and more than 50% of asthma exacerbations in children are caused by a viral infection.[1-6] The burden of disease in adults is equally high: viruses are implicated in 30-50% of patients with community acquired pneumonia (CAP) and in more than 80% of asthma exacerbations.[5]

Influenza virus

Influenza is the only respiratory virus for which effective antiviral therapy and vaccination are available. However, the continuously changing genetic composition of the virus, leading to 'antigenic drift', forces frequent renewal of vaccines and hinders the development of long-lasting protective immunity. During every replication minor changes in the RNA can occur. This antigenic drift is due to the fact that the RNA-dependent RNA polymerase of the virus has an average of one error per replication in the absence of RNA proofreading enzymes.[7,8] Due to the segmented nature of the influenza A, genome reassortment of hemagglutinin (H) and sometimes neuramidase(N) genes, can occur between co-infecting Influenza strains. This abrupt and major change is called an 'antigenic shift'.[8-10] Reassortment between H and N genes of 2 (or more) different influenza strains occur through mixture of these strains in a susceptible host (human, avian, swine). Every antigenic shift event has the potential to cause a pandemic.[8,10,11]

In **Chapter two** the clinical role of emerging respiratory viruses in the past two decades is discussed. The introduction of PCR and sequencing techniques enabled rapid detection of novel viruses as compared to slower traditional techniques such as viral culture. However, not all viruses are completely characterized according to the (modified) Koch's postulates.[12,13] In some cases, such as Bocavirus, the pathogenicity of the virus is not fully elucidated since this virus is also often detected in asymptomatic children.[12-15] Since our publication in *Hot Topics in Infection and Immunity in children IX* several novel respiratory viruses have emerged.[16] Examples are the MERS coronavirus (Middle East respiratory syndrome) in 2012, the novel influenza A H7N9 virus in 2013 and novel outbreaks of enterovirus (EV) D68 infections in 2014.[16] MERS is caused by an RNA βcoronavirus that was first isolated from a patient in 2012.[17-19] The virus is thought to spread through dromedary camels.[20] Infection can be asymptomatic or mild, but is often severe and results in acute respiratory distress syndrome and multi-organ failure.[21-24] The case fatality rate is 40% in the general population and up to 60% in (elderly) patients with co-morbidities such as diabetes or cardiovascular disease. The

median age of patients is 50 years [range 1 -94 years]. Approximately 2% of the reported infections are in children, often with a relatively mild course of disease.[21-25] Human to human transmission has been described, especially during hospital outbreaks.[23] The virus is not (yet) adapted to the human host, limiting its efficiency in transmission and therefore its pandemic potential.[19] Prevention of infection is the most important intervention, since there is no treatment or vaccine against the disease.[25] In 2013 a novel Influenza A H7N9 virus emerged in China.[19] Infections with this virus were associated with typical influenza symptoms, such as fever, cough, chest pain and pneumonia. Severe cases experience respiratory failure, acute respiratory distress syndrome, multi organ failure and/or encephalopathy. Influenza A H7N9 has a mortality rate of 39%, mainly in patients older than 60 years of age. [19,26] In patients below 60 years infections appear to resolve sooner and are often less severe.[27] The mode of transmission is poorly understood. Birds are a potential source of infection since reductions in the number of new cases coincided with closure of poultry markets.[19] Human to human transmission cannot be excluded.[28]

Enterovirus D68 (EVD68), a member of the Picornaviridae family was first isolated in 1962 in children with pneumonia and bronchiolitis.[29] Since 1962 EVD68 was occasionally detected as a cause of severe respiratory illness.[30,31] EVD68 spreads in humans by close contact with infected people. In 2014 clusters of (severe) respiratory disease and asthma exacerbations coincided with children with acute flaccid paralysis. It appeared that the respiratory and neurologic problems were both caused by EVD68. [32-34] Since then EVD68 is also more frequently detected in other countries.[31,35]

Monitoring of zoonotic viruses (camel MERS-Cov, avian Influenza A H7N9, avian Influenza A H10N8, swine influenza A H3N2) is difficult since infections do not always cause significant health problems in these animals. The limited understanding of the pathogenesis and transmission of these novel pandemic and lethal viruses warrants global alertness and intensive surveillance to enable early detection.[36] Surveillance networks have contributed to a rapid detection and identification of the pandemic Influenza A H1N1 virus in 2009 and the prevention of this infection by vaccination.

In **Chapter 3** we describe the clinical symptoms and course of disease in Dutch children who were hospitalized for infection by influenza A H1N1. We observed that the majority of patients was older than 5 years and suffered from an underlying medical condition. However, the most severe and acute presentations occurred in previously healthy children below the age of 3 years. In **Chapter 4** clinical signs and symptoms of hospitalized children with PCR proven H1N1 influenza A infections are described as part of a nationwide retrospective study in the Netherlands. Groups at risk for a severe course of disease were identified. There were strong similarities between these risk groups and those previously reported for seasonal influenza.[37-39] Especially children with neurological conditions and psychomotor retardation were at high risk for a severe outcome. The H1N1 influenza A virus that emerged during the pandemic continues to circulate worldwide and remains a major reason for ICU admission in both adults and children with and without risk factors. [40,41] A sudden onset and a severe course of

disease in previously healthy children have been documented during both pandemic and seasonal influenza infections. This observation is striking, since severe disease is mainly expected in high risk groups that are eligible for vaccination.
The European Centre for Disease Control (ECDC) has pointed out that additional studies on risk groups for severe influenza are necessary before routine vaccination of children can be recommended.[42,43] In **Chapter 5** the criteria of the Dutch Health Council for introduction of vaccines in the national immunization program (NIP) were used to analyze whether influenza vaccination should be introduced in the NIP.[44] In this review from 2013 we concluded that not all criteria were met. Recently, Cromer *et al.* showed that risk groups remain at risk for severe disease even after they are vaccinated.[45] Moreover, 40% of physician consultations and hospital admissions occurs in children under 15 year old. This indicates that the current risk group-based vaccination strategy does not protect this age group of healthy children.[45] Several arguments favor general vaccination of children. Children are key participants in the spread of influenza, which is due to close contacts between children at schools and to immunological factors, such as an immature adaptive immune system, leading to prolonged shedding. [46-49] Vaccination of all children against influenza instead of only risk groups does also protect non-vaccinated individuals by means of herd immunity. [50-52] Mathematical modelling using data of 14 influenza seasons in England show that vaccination of all children between 5-16 years is beneficial and cost-effective.[53] The outcome of this mathematical analysis indicates that the vaccination of primary or secondary school aged children is highly cost effective. A combined vaccination program for primary and secondary school aged children provides an optimal strategy.[54] Currently, seasonal influenza vaccination with a quadrivalent nasal live attenuated influenza vaccine (LAIV) is part of the NIP in England. [42,43,55] Initial reports have shown encouraging results indicating that both direct and indirect protection occur, resulting in a decreased incidence of influenza like illness and of upper and lower respiratory tract infections.[56-58] To date, influenza vaccination for children is gradually rolled out over more areas in the UK.
There is no influenza vaccine available for children below 6 months of age, although the burden of disease is highest in children in this age group.[45,59,60] Maternal vaccination may reduce the burden of disease by influenza in infants <6 months of age. Maternal vaccination is safe and recommended by the WHO since 2012.[61] A recently published study on maternal vaccination in the 2013/2014 season showed an effectiveness of 71% to prevent influenza in infants younger than 6 months and an effectiveness of 64% to prevent hospitalization.[62] This is in agreement with a previously published randomised controlled trial that produced similar results: 63% vaccine effectiveness and 29% reduction in periods of respiratory illness with fever.[63]

Conclusions and future perspectives on part I of this thesis

Children at risk for severe influenza A H1N1 infections are also at risk for infection with seasonal influenza. A large proportion of hospitalized children, including those with severe disease, were previously healthy. The evidence that vaccination of all children

would lead to protection in the whole population was in 2013 not strong enough to meet the criteria of the Dutch Health Council to advise seasonal influenza vaccination for all children. However, the data on the burden of influenza disease in infants, the cost-effectiveness of vaccination of healthy children and the preliminary results of influenza vaccination of children in the national immunization program in the United Kingdom suggest that the Netherlands should also consider to provide influenza vaccination to all children. Before doing this, It will be necessary to study the public perception regarding influenza vaccination in the Netherlands and to educate the public about the advantages and disadvantages of use of this vaccine in children. This may alleviate safety concerns regarding influenza vaccination, which otherwise may be a reason for refusal.[64-66] The Netherlands have a high uptake rate of influenza vaccination in high risk groups (81%).[67,68] However, after the introduction of the HPV vaccination in young girls there was much debate in social media, politics and the scientific community regarding the safety, duration of protection and the age at which the vaccine was given, which illustrates the importance of appropriate information and education before and during the introduction of new vaccines.[69,70] Moreover, the logistics of an extended influenza vaccine program will be challenging due to a yearly need for a vaccine which is adapted to the circulating seasonal influenza strain. Studies from the UK and the US show that school-based vaccination is a successful model resulting in higher uptake as compared to vaccination by general practitioners and health care centers.[56,57] In addition, maternal vaccination is an effective way to reduce the burden of disease in children younger than 6 months. Maternal vaccination is already advised in several European countries.[71,72] Studies are needed to assess whether routine vaccination of healthy children also reduces the burden of influenza in children less than 6 months of age via herd immunity. If this is true, we suggest to give higher priority to the introduction in the NIP of maternal vaccines against *Bordetella pertussis* and RSV, as compared to maternal vaccination against influenza.

Respiratory Syncytial virus (RSV)

RSV was discovered over sixty years ago. Infections by RSV are considered as a major burden of disease in children worldwide.[73,74] To date there is no antiviral treatment available and vaccines against RSV are still under development. Administration of palivizumab, a prophylactic monoclonal antibody, provides passive immunization to high risk groups, but is not available for all children due to high costs. Moreover, palivizumab has little effect once patients are infected by RSV and is not useful as treatment of severe RSV infections.[75,76] Therefore treatment of infections by RSV is limited to supportive care consisting of respiratory support and adequate hydration.

The pathophysiology of disease by RSV is complex. Direct cytopathic effects of the virus and enhanced inflammatory responses are important in the pathogenesis of disease.[77] Hospitalization for RSV bronchiolitis is necessary in approximately 1-3% of infants and children.[78] The severity of disease in young infants can vary from mild upper respiratory tract infections to respiratory failure. The underlying cause for differences in

severity of disease still needs to be elucidated.[79-81] Risk factors for a severe course of disease, such as prematurity, immunodeficiencies and congenital heart and lung disease are well described.[82] However, RSV infection leads to a remarkable variability in level of severity. More than half of the young children in the ICU are otherwise healthy.[83] Clinicians use clinical parameters and known risk factors to predict the level of severity and the course of disease to assess once a patient with bronchiolitis should be admitted to the hospital. However, this assessment has limitations, since approximately one third of hospitalised patients do in retrospect not require supportive treatment, whilst 4.6-6.8% of children who were sent home needs to be admitted later in the course of disease.[84-86]

Biomarker discovery

In **Chapters 6-10** we describe several biomarkers that can differentiate between different levels of disease severity of children with viral RTIs. In **Chapters 6-8** we studied whether the biomarkers that were described in the context of (chronic) lung disease and immune responses against bacterial LRTI can be used to determine viral LRTI disease severity.

In **Chapter 6** we demonstrated that a combination of inflammatory proteins PTX3, CRP, SAA and Properdin can discriminate patients with a severe course of disease from children with a moderate course of viral disease. The area under the curve (AUC) of this combination of protein levels was 0.89.[87,88] In a validation cohort of children with viral LRTI the test had a sensitivity and specificity of 71% and 87%, respectively. This study shows that a strategy to combine known inflammatory markers may be an attractive approach to optimize the performance of individual laboratory tests as biomarkers for disease severity. Measurement of PTX3, CRP, SAA and Properdin at an earlier stage of disease is necessary to test their true predictive value. In addition, the performance of these markers should be investigated in other paediatric infectious diseases.

In **Chapter 7** we studied the expression of surface markers on circulating monocytes during RSV infection and correlated the outcomes with disease severity. Monocytes are important innate immune cells in antiviral immunity.[89-91] Circulating monocytes can be divided in three groups (classical, intermediate and nonclassical), based on their expression of CD14 and CD16.[92] We demonstrated that significant shifts in monocyte subpopulations occurred during acute RSV infections. Changes included the expression of surface proteins CD14 and CD16, and the increased expression of HLA-ABC and decreased expression of HLA-DR. Decreased expression of HLA-DR on monocytes was correlated with disease severity. This phenomenon has also previously been observed during bacterial infections.[93-95] Whether this may be of prognostic value, as was shown for septic neonates, is not yet clear.[93] PBMCs of infants were stimulated with LPS *in vitro.* Furthermore, cytokine responses were measured in healthy controls and RSV infected patients with a severe and non severe course of disease. Patients with a severe RSV infection had normal TNF responses, but showed a decreased IL-10 production when supernatant levels were compared to the responses of healthy

controls. This contradicts with previously observed 'immune paralysis', a phenomenon that is characterised by low HLA-DR expression and decreased TNF responses.[93-95] We therefore assume that RSV does not induce the classical immune paralysis during severe infection, although an imbalanced immune response may play an important role in severe disease.

Increased serum concentrations of MMP8 and MMP9 are present during acute lung injury and pneumonia in adults and children.[96-98] MMP8 and MMP9 proteins can degrade the extracellular matrix, aid cell migration of inflammatory cells and have immunomodulatory capacities.[99,100] MMP 9 levels are correlated with severity of asthma and pneumonia. [96,97] In **Chapter 8** MMP 8 and MMP 9 protein levels and gene expression were measured in plasma and nasopharyngeal aspirates to see whether they can serve as a biomarker for severity of RSV disease.[100] We observed some differences in plasma levels of MMP8 and MMP9 between children with mild and severe disease. However, the clinical value of MMP8 and MMP9 measurements as protein biomarkers for RSV disease severity is limited due to a broad overlap between the groups. In contrast, measurement of gene expression levels of MMP 8 and MMP 9 in both PBMCs and granulocytes may have more potential to be used as a future biomarker. MMP8 levels were differently expressed in granulocytes from patients with moderate and severe disease and in PBMCs from patients with mild and moderate disease. MMP 9 gene expression levels were also differently expressed in granulocytes from mildly versus moderately infected patients and in PBMCs from patients with moderate and severe disease. Moreover, a subsequent study demonstrated the potential of MMP9 as biomarker for severity of RSV infections in infants.[101] I*n vitro* studies on human airway epithelial cells demonstrate that RSV is a potent stimulus for MMP9 gene expression and release and that MMP9 can prevent RSV infection of human airway epithelial cells *in vitro* and infection of mice lungs. [101-103] In MMP9 knock-out mice neutrophil recruitment and cytokine production is significantly enhanced upon RSV infection.[103] Kong *et al.* demonstrated that MMP9 knock-out mice or mice treated with siRNA to silence MMP9 gene expression had less lung inflammation and decreased neutrophil influx upon RSV infection and decreased viral replication.[102] They propose that excess MMP9 activity may also serve as novel target for therapeutics.[102]

Micro-array studies

In **Chapter 9** we performed microarray analysis on the PBMC's of children during a primary RSV infection. By using on a Prediction Analysis of Microarrays (PAM), Olfactomedin 4 was found as the sole marker that identified patients with severe disease with 0% cross-validation error. This finding was validated in another cohort of infants with RTI (both RSV positive and negative). The relative risk for de development of severe disease in children with a high OLMF4 level was 6.1, after correction for age and preterm status. We also observed that plasma levels of Olfactomedin 4 were not as discriminative as gene expression levels, which is in agreement with the data presented in **Chapter 8**. This indicates that analysis of gene expression levels in peripheral blood may be more

sensitive and suitable to determine severity biomarkers than by measurement of protein levels. Furthermore, we concluded, on the basis of data from other micro-array studies in children with viral infections, that the Olfactomedin 4 response is not RSV-specific. Olfactomedin 4 may therefore also be a marker for disease severity in other types of infections. Thus far the biological function of OLFM4 is not yet completely understood. Studies have mainly focused on its role in cancer, but it appears that OLFM4 also plays a role in innate immunity. It has been shown that during infections with *H. pylori* OLFM4 may down-regulate the NF-*k*B pathway.[104,105] In addition, OLFM4 deficient mice exhibit enhanced immunity against sepsis and infection by *Escherichia coli* and *Staphylococcus aureus*.[106] This indicates that OLFM4 is an important negative regulator of neutrophil bactericidal activity by influencing cathepsin C activity.[106,107] OLFM4 is a protein which is stored in specific granules of a subset of circulating human neutrophils (20–25 %).[108] However, until now no difference in OLFM4-positive and OLFM4-negative neutrophil functionality could be demonstrated. Interestingly OLFM4 is released in Neutrophil extracellular traps (NETs), but whether this elicits a different function or effect is not known.[108,109]

Gene expression studies on PBMCs require a cell isolation step, which is time consuming and hinders application in the clinical setting. Therefore, we adapted protocols to measure gene expression on whole blood. First we investigated whether a gene signature in whole blood could be identified that predicts the course of RSV infection in infants with high accuracy.

In **Chapter 10** whole blood gene expression data was corrected for age and gender (two confounders for disease severity) and analyzed with different bioinformatic approaches, including machine learning to optimize biomarker discovery**.** We identified an expression profile of 84 genes in whole blood which distinguished the patients with severe RSV disease with good performance. This set of 84-genes was validated in an independent cohort (that used the Illumina microarray platform instead of the Affymetrix platform) and showed an AUC of 0.856 to identify patients with a severe RSV disease. The exact clinical value should be assessed in future studies. Transcriptional profiling can be used to separate bacterial from viral disease. Ramilo *et al.* were among the first that showed differences in the host response between children with viral and bacterial infections, and also differences in gene expression between the different viruses and bacteria.[110,111] Herberg *et al.* recently published a validated two gene signature which allows differentiation between bacterial and viral causes of disease in children with fever.[112] This two transcript RNA signature (*FAM89A* and *IFI44L)* has a high sensitivity and specificity (≥90%).[112] A similar study was published simultaneously about infants with fever below 60 days of age, for which a gene set that consisted of 10 genes (including MMP9) was used. It was shown in a validation cohort that infants with serious bacterial infections could be identified with a sensitivity and specificity of >90%.[113]

Another method to analyze transcriptome data is by measurement of the distance to health.[111] Distance to health compares the total gene expression of a patient having a certain disease, i.e. viral RTI, with the total gene expression of healthy infants and

compiles these differences to one score. In **Chapter 11** we show that distance to health in children with RSV LRTI correlates with disease severity, length of hospitalization and duration of supplemental oxygen. This method has also been used in other studies and appears to be a good marker to 'quantify' disease severity during infectious diseases, such as tuberculosis and in children with RSV or *Staphylococcus aureus* infections.[111,114,115] The analysis of distance to health seems to offer a promising conceptual approach to study disease severity in infectious diseases. It is interesting to investigate whether this is also true for other non-infectious diseases.

Chaussabel *et al.* developed a modular analysis method for micro-array studies that enabled the study of co-expressed genes instead of single genes. This provides a novel way to study the behavior of cell types and processes during infections.[116] In **Chapter 11** this method was adapted to the Affymatrix micro-array platform and applied to whole blood transcriptome profiles in order to improve the understanding of the pathophysiology of severe RSV disease. Six modules were identified that had significant differences in expression across the disease severities. The neutrophil module showed the most apparent difference between the severity groups, with significantly higher values in infants with severe disease compared to those with a mild and moderate course of disease. Cytotoxic T-cells and NK cells modules were downregulated during severe RSV infections. This was probably a result of lower cell counts as previously shown by our group.[117] The modules on inflammation, platelets and erythrocytes were upregulated. Similar results were seen in another, more extensive study that compared the immune response against different viruses, but also between RSV disease severities.[111]

The role of neutrophils during viral infections is intriguing and not well understood. In infants with severe disease a strong influx of neutrophils is observed in bronchoalveolar lavages and an accumulation of neutrophils is seen in lung tissue and mucus plugs of the airways in autopsies of infants who deceased during severe RSV infections.[118,119] It appears that neutrophils play an important role in airway obstruction, a hallmark of severe RSV infection.[76] Recently, several studies have elucidated that neutrophils have antiviral capacities.[120,121] Neutrophil extracellular traps (NET) are induced by RSV and can prevent RSV from infecting new cells. However, an exaggerated formation during severe disease also contributes to disease severity.[121] Iversen *et al.* demonstrated that an innate antiviral pathway can be activated by epithelium, which attracts neutrophils prior to the interferon response.[120] NETs have been investigated in the context of other viral infections, such as influenza virus and appear to play a role in the containment and prevention of viral infection.[122] [123] It is intriguing that MMP8, MMP9 and OLFM4-markers that correlate with RSV disease severity-are stored in the granulae of neutrophils. [108,124]

Mucosal biomarkers

In the previous chapters we chose to study the systemic effects of a local viral infection, because it is difficult to obtain material from the lower respiratory tract from young children. Analysis of parameters in peripheral blood provides a reflection of disease

severity as was shown in the preceding part of this thesis. However, it is thought that the local inflammatory response may initiate the development of severe disease. We therefore further explored the inflammatory response at the mucosal level. A nasopharyngeal aspirate (NPA) is relatively easy to obtain and can be a source for potential biomarkers.

In **Chapter 12** we show that we can successfully perform microarray analysis on NPA (containing epithelial cells and immune cells). This analysis revealed several genes, such as TSPAN 8, MUC13, MSP and CCL7 that were differently expressed between patients with mild or moderate and severe disease. These results were validated with qPCR in an independent cohort and delivered similar results. We believe that transcriptional analysis of NPA specimens is a promising approach for diagnostic procedures, which can relatively easy be combined with pathogen detection. This technology platform also offers the opportunity to establish a microbiological diagnosis and at the same time provide the clinician with insight in (the prognosis of) severity of disease.

Mucosal immune responses

Viral infections of the respiratory tract always occur in the presence of colonizing bacteria (microbiome). Bacterial co-infection cannot always be excluded in patients with severe viral disease.[125] In our studies on viral RTIs routine cultures did not reveal many bacterial co-infections. Most children with viral RTIs that were not admitted to the ICU did not receive antibiotics, indicating that in non-mechanically ventilated children bacterial co-infections do not play an important role. The effect of the colonizing bacteria on the anti-viral host response in the respiratory tract has not yet been studied extensively. However, interactions between enteric viruses, antiviral immunity and the gut microbiome receive increasing interest.[126-128]

In **Chapter 13** we found that the presence and density of *Streptococcus pneumoniae* and *Haemophilus influenzae* in the nasopharynx of patients with RSV infections did not correlate with viral load, inflammation or severity of disease. However, when patients were colonized with *Streptococcus pneumoniae*, the bacterial density correlated with an increased RSV load, higher MMP 9 levels and a decreased disease severity, indicating that the presence of bacteria can indeed affect the anti-viral host-response. Recently De Steenhuijsen Piters *et al.* reported that the nasopharyngeal microbiome interacts with the immune response during RSV infection and may influence disease severity. [129] They demonstrated that an upper respiratory tract microbiome enriched for *H. influenzae* or *S. pneumoniae* was associated with an increased disease severity defined as a need for hospitalization. In modular gene expression analysis of peripheral blood from patients with these enriched microbiomes, the responses related to TLR-signaling and neutrophil recruitment were more pronounced. This confirms the hypothesis that colonization is associated with modulation of the immune response during RSV infections. This phenomenon has also been reported during influenza virus infections and may indicate that microbiota regulate immune defense during respiratory viral infections and calibrate the activation threshold for (innate) immune responses.[128,130]

Suarez-Arrabal *et al*. demonstrated that colonization with Gram-negative bacteria (*Moraxella catarrhalis* and *H. Influenzae*) results in increased numbers of mucosal and systemic inflammatory cells, higher pro-inflammatory cytokine levels and a trend towards increased disease severity.[131] This is in agreement with the study from De Steenhuijsen Piters *et al.,* but in contrast with the results presented in **Chapter 13**. This may be due to differences in study protocols and detection methods. We used a 16S qPCR specific for *Streptococcus pneumoniae* while in the other studies 16S rRNA gene amplicon sequencing was used, by which the different streptococcal species cannot be distinguished. In addition, inflammation was in our study defined by protein levels, whereas De Steenhuijsen Piters *et al.,* used gene expression. As shown in chapters 8 and 9 these do not always correlate.

The respiratory mucosa and the mucus contain antibodies. In general, immunoglobulins directed against RSV are thought to be protective, as reflected by the reduction of disease severity by passive immunization with palivizumab and protection against RSV by high maternal antibodies.[132-135] The RSV vaccination trial in the sixties, where immunization led to more severe disease and mortality, illustrates that levels of antibodies do not always correlate with protection, because a strong antibody responses upon vaccination were measured. [136] Although the exact mechanism of 'antibody enhanced disease' has not yet been clarified, titers of non-neutralizing antibodies and the level of avidity of antibodies seem to play an important role in RSV disease.

In **Chapter 14** we show that there is no correlation between the plasma levels and mucosal levels of IgG in children with viral RTI's. Levels of mucosal IgG correlate better with viral load and mucosal inflammation than levels of plasma immunoglobulin. This finding can be of importance for the evaluation of future vaccination studies in children. It may also be an argument for intranasal vaccination in children. A relation between better local CD8+ T cell responses after nasal vaccination compared to intraperitoneal vaccination has already been demonstrated by Morabito *et al.* in mice.[137]

Conclusions and future perspectives on part II of this thesis

The clinical value of biomarkers which we have identified should be assessed in prospective studies that include serial measurements and should ideally start from an earlier time point in the course of disease, preferably at the initial visit to the general practitioner. Thus far only a restricted number of analyses could be performed per patient due to a limited amount of blood that can be taken from young infants. Due to improved techniques the amount of blood per test is dramatically reduced (we used 3 ml blood for a micro-array analysis while this test is now possible with 1.0 ml).[138] In this way, more markers can be tested simultaneously, reducing the ethical and safety arguments against the assessment of different time points. When validation studies show similar results, also at earlier time points, the markers can be used in a clinical trial. At that point most of the criteria for a good biomarker as proposed by Morrow and de Lemos have been fulfilled.[139] These criteria state that a good biomarker should be easy to measure and add information to the clinical decision progress. Importantly, a

good biomarker should have an impact on clinical management.[139] Sequential testing can have additional value, since study of the kinetics of biomarkers may provide additional useful information. Furthermore, the use of a combination of biomarkers may increase the sensitivity and the specificity of these tests as described in Chapter 6. Once an optimal gene set or protein panel is established it should be converted to a point-of-care test. Techniques for bed-side PCR and protein measurement in plasma are already available.[140,141]

Future studies should assess whether expression profiling of a limited number of genes can also be used to reflect distance to health and assess the potential of implementation of this maker in clinical practice. In addition, the modular analysis developed by Chaussabel *et al.* can be used to gain more insight into the pathophysiology of severe RSV infections, but may also be applied to other (infectious) diseases. The combined measurement of genomic data, microbiome analysis and expression profiling, may enhance the understanding of the interaction between the bacterial microbiome and the human immune response, especially when used in a human experimental challenge model. Integration with other 'omic' techniques, such as metabolomics and proteomics, will facilitate in-depth analysis of the host-pathogen interaction and offers the opportunity to decipher individual susceptibility for severe RSV-infections.[142]

Several antivirals are currently under investigation. At this moment only ribavirin is approved for the treatment for RSV.[143] The use of ribavirin in severe RSV infections is controversial and not recommended. It is difficult to administer ribavirin in ventilated patients in the ICU and the drug is expensive.[143,144] Potential new antivirals can be divided into three groups based on their mechanism of action, 1.) nucleocapsid protein inhibitors (RSV604), 2.) RNA-dependent RNA polymerase inhibitors (YM-52403, BI-DD and ALS-8176) and 3.) fusion inhibitors (TMC-353121, BMS-433771, VP-14637 and GS-5805).[145-149] Currently, only ALS-8176 and GS-5806 are tested in clinical trials. [150,151]

By revealing the crystal structure of the F-glycoprotein of RSV in its pre-triggered (prefusion) conformation the RSV vaccine development has exhilarated.[152,153] Neutralizing antibodies mainly bind to the prefusion form of the F protein (preF).[154,155] This preF is highly unstable and easily switches to its postfusion form (postF), which has no function in fusion of membranes and viral entry into a host cell.[156,157] Therefore, antibodies directed against the postF offer limited protection. The development of monoclonal antibodies targeting preF is more effective in reducing viral infection.[155,158] Once the half-life of these antibodies can be increased they could be used for passive immunization of which only one administration of these antibodies will be enough to reduce disease severity in the first 6 months of life.

Intranasally administered live-attenuated vaccines or chimeric live vector vaccines, are currently tested in clinical trials and seem to be safe for children older than 6 months of age.[159-162] Further, gene-based replication-defective vaccines with viral vectors or nucleic acid are considered safe and are in early phases of clinical testing in adults.[159,160] Subunit proteins and protein based nanoparticles are proposed for maternal vaccination purposes to protect the youngest children.[160] Other vaccine strategies, such as virus-like

particles and virosomes, have been tested in animal studies, but not yet in clinical trials.[159] The current clinical trials on antivirals and vaccines offer the potential for future generations to change the epidemiology of RSV disease. Availability of validated biomarkers to predict disease severity of viral RTI will help to design studies to assess antivirals and vaccines and thus will contribute to decrease the number of patients with severe RSV disease.

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16

Appendices

Nederlandse samenvatting
Curriculum Vitae
Dankwoord
Publicaties

Introductie

Acute lage luchtweginfecties (LLWI's) zijn de meest voorkomende oorzaak van ziekte bij kinderen en de belangrijkste oorzaak van sterfte in de leeftijdsgroep van twee maanden tot vijf jaar. Mortaliteit ten gevolge van onderste luchtweginfecties (OLWI) is zeldzaam in de westerse wereld, maar nog steeds aanzienlijk in delen van Afrika, Azië en Zuid-Amerika. Elke 35 seconden sterft er ergens op de wereld een kind aan een lage luchtweginfectie, de 3FM Serious Request actie van 2016 zamelde geld in en bracht dit onderwerp onder de aandacht van het grote publiek. Virussen werden voorheen als minder relevant beschouwd in vergelijking met bacteriën als veroorzakers van ernstige lage luchtweginfecties. Tegenwoordig worden virussen gezien als belangrijke pathogenen bij deze aandoening bij volwassenen als kinderen. Meer dan 90% van de kinderen met bronchiolitis, ruim 50% van de kinderen met longontsteking en astma exacerbaties wordt veroorzaakt door een virus. [1-6] De ziektelast bij volwassenen is eveneens hoog: virussen zijn aanwezig bij 30-50% van de patiënten met een in de gemeenschap verworven pneumonie (CAP) en in meer dan 80% van de astma-exacerbaties.[5]

Influenza virus pH1N1

Influenza is het enige respiratoire virus waarvoor effectieve antivirale medicatie en vaccinatie beschikbaar is. Doordat de genetische samenstelling van het influenza virus telkens verandert door "antigene drift", moeten de vaccins jaarlijks aangepast worden. Dit is de reden waarom er geen goede immuniteit bij de mens ontstaat. Antigene drift ontstaat doordat bij elke replicatie van het virus gemiddeld 1 fout gemaakt wordt door het RNA-afhankelijke RNA-polymerase. Aangezien het influenza virus geen correctie enzymen heeft die deze fout kunnen corrigeren, verandert het virus bij elke deling een beetje en ontstaat er uiteindelijk een virus dat niet meer herkend wordt door de aangemaakte antilichamen na een vaccin of ziekteperiode.[7,8] Als meerdere influenza stammen een zelfde host (mens, vogel, varken) infecteren, kan herschikking van hemagglutinin (H) en soms neuraminidase (N) genen optreden. Dit is mogelijk doordat het genoom van het influenza A virus gesegmenteerd is. Een dergelijke abrupte en grote verandering in het genetisch materiaal heet een 'antigene shift'. [8-10] Elke antigene shift heeft de potentie om een pandemie te veroorzaken.[8,10,11]

In **hoofdstuk 2** van dit proefschrift zijn de nieuw ontdekte respiratoire virussen van de afgelopen twee decennia besproken. Door de invoering van PCR- en sequencing technieken worden er veel meer virussen ontdekt dan voorheen. Met de traditionele technieken, zoals o.a. viruskweek, was dit moeilijker en tijdrovender en niet alle virussen kunnen zo ontdekt worden. De meeste van de recent geïdentificeerde virussen zijn echter nog niet volledig gekarakteriseerd volgens de (gemodificeerde) Koch's postulaten. [12,13] In sommige gevallen, zoals bij het bocavirus, is de pathogeniciteit van het virus niet volledig vastgesteld, aangezien dit virus ook wordt aangetroffen bij asymptomatische kinderen. [15-12] Sinds onze publicatie in *Hot Topics in Infection and Immunity bij kinderen IX* zijn verschillende nieuwe respiratoire virussen ontdekt. [16]

Voorbeelden zijn MERS coronavirus (Middle East Respiratory Syndrome-coronavirus' (MERS-CoV) in 2012, het nieuwe influenza A H7N9-virus in 2013 en nieuwe uitbraken van enterovirus (EV) D68 infecties in 2014. [16] MERS-CoV wordt veroorzaakt door een RNA-β-coronavirus dat in 2012 voor het eerst werd ontdekt bij een zieke patiënt. [17-19] Dromedarissen lijken de verspreiders van het virus. [20] Een infectie kan asymptomatisch of mild verlopen, maar kent ook vaak een ernstig beloop leidend tot *acute respiratory distress syndrome* en multi-orgaanfalen. [21-24] De letaliteit is 40% in de algemene bevolking en oplopend tot 60% bij (oudere) patiënten met co-morbiditeiten, zoals diabetes of hart- en vaatziekten. De gemiddelde leeftijd van patiënten is 50 jaar [range 1 -94 jaar]. In 2% van de gerapporteerde infecties betreft het kinderen, vaak met een relatief mild ziekteverloop. [21-25] Van mens op mens overdracht is beschreven, vooral tijdens ziekenhuisuitbraken. [23] Het virus is (nog) niet aangepast aan de menselijke gastheer, hierdoor is de efficiëntie van verspreiding laag en de kans op een pandemie beperkt. [19] Preventie van infectie is de belangrijkste interventie, omdat er geen behandeling of vaccin tegen de ziekte is. [25]

In 2013 ontstond een nieuwe Influenza A H7N9 virus in China. [19] Infectie met dit virus is geassocieerd met typische influenza symptomen, zoals koorts, hoesten, pijn op de borst en longontsteking. Ernstige gevallen ervaren respiratoire insufficiëntie, *acute respiratory distress syndrome*, multi-orgaanfalen en/of encefalopathie. Influenza A H7N9 heeft een letaliteit van 39%, vooral bij patiënten ouder dan 60 jaar. [19,26] Bij patiënten jonger dan 60 jaar lijken infecties een kortere duur te hebben en verlopen ze vaak minder ernstig. [27] De wijze van overdracht is nog onduidelijk. Vogels zijn een potentiële bron van besmetting aangezien vermindering van het aantal nieuwe gevallen samen viel met sluiting van pluimvee markten. [19] Mens op mens overdracht kan niet worden uitgesloten.[28] Enterovirus D68 (EVD68) is een lid van de familie Picornaviridae. Het werd voor het eerst geïsoleerd in 1962 bij kinderen met een longontsteking en bronchiolitis. [29] Sinds 1962 werd EVD68 zo nu en dan gedetecteerd als een oorzaak van ernstige ziekte van de luchtwegen. [30,31] EVD68 verspreidt zich door nauw contact met besmette patiënten. In 2014 werden clusters van (ernstige) aandoeningen van de luchtwegen en astma exacerbaties gerapporteerd, deze vielen samen met berichten over kinderen met een acute slappe verlamming. Het bleek dat de ademhalings- en neurologische problemen beide veroorzaakt werden door EVD68. [32-34] Sindsdien wordt EVD68 wereldwijd vaker waargenomen. [31,35]

Screenen op nieuwe zoönotische virussen (dromedaris MERS-Cov, aviaire influenza A H7N9, aviaire influenza A H10N8, varkens influenza A H3N2) is moeilijk omdat infecties door deze virussen bij dieren meestal geen waarneembare gezondheidsproblemen veroorzaken. De beperkte kennis van de pathogenese en de transmissie van deze nieuwe pandemie en dodelijke virussen maakt wereldwijde alertheid noodzakelijk in combinatie met intensief toezicht om vroege opsporing mogelijk te maken. [36] Surveillancenetwerken hebben bijgedragen tot een snelle detectie en identificatie van de pandemische influenza A H1N1-virus in 2009 en de preventie van deze infectie door vaccinatie.

In **hoofdstuk 3** beschrijven we de klinische symptomen en het beloop van ziekte bij Nederlandse kinderen die opgenomen werden vanwege de pandemische influenza A H1N1 infectie in het academisch ziekenhuis in Nijmegen. De meeste patiënten waren ouder dan 5 jaar en hadden een onderliggende medische aandoening. Echter, de meest ernstige en acute presentaties vonden plaats bij voorheen gezonde kinderen jonger dan 3 jaar waren. Het Europees Centrum voor ziektepreventie en -bestrijding (ECDC) heeft erop gewezen dat aanvullende studies naar risicogroepen voor ernstige griep nodig zijn alvorens routinematige vaccinatie van kinderen kan worden aanbevolen. [42,43]
In **hoofdstuk 4** zijn de klinische symptomen van (bijna) alle gehospitaliseerde kinderen met een PCR bewezen H1N1 influenza A-virus infectie beschreven in een Nederlands retrospectief onderzoek. De risicogroepen voor een ernstig beloop zijn op basis hiervan geïdentificeerd. Er bleken sterke overeenkomsten tussen deze risico-groepen tijdens de H1N1 pandemie en de risicogroepen van de gewone seizoensgriep. [37-39] In het bijzonder kinderen met neurologische aandoeningen en psychomotore retardatie hadden een hoog risico op een ernstig beloop. Het H1N1-influenza A-virus dat deze pandemie veroorzaakte, blijft wereldwijd circuleren en blijft een belangrijke reden voor IC opname bij zowel volwassenen als kinderen met en zonder risicofactoren. [40,41] Een plotseling begin en een ernstig verloop van de ziekte bij voorheen gezonde kinderen zijn gedocumenteerd, zowel tijdens de pandemie als bij de seizoensgebonden influenza-infecties. Deze waarneming is opvallend, aangezien ernstige ziekte vooral wordt verwacht in een hoog risico groepen die in aanmerking komen voor vaccinatie.
In **hoofdstuk 5** zijn de criteria die de Nederlandse Gezondheidsraad hanteert voor toelating van vaccins tot het Rijksvaccinatieprogramma (RVP) gebruikt om te analyseren of influenzavaccinatie in het RVP moet worden ingevoerd. [44] In dit review uit 2013 hebben we geconcludeerd dat het onderzoek naar influenza vaccinatie nog niet aan alle criteria voldoet. Recentelijk hebben Cromer *et al.* laten zien dat patiënten uit risicogroepen ondanks vaccinatie nog steeds at risk zijn voor een ernstig ziektebeloop. [45] Ook blijkt dat 40% van de doktersbezoeken en ziekenhuisopnames in verband met influenza infecties voorkomt bij kinderen onder de 15 jaar. Dit geeft aan dat de huidige risico-groep-gebaseerde vaccinatie strategie deze leeftijdsgroep van gezonde kinderen onvoldoende beschermd. [45] Er zijn verschillende argumenten ten gunste van algemene vaccinatie van kinderen: kinderen zijn de belangrijkste bron voor de verspreiding van influenza, enerzijds door het intensieve contact wat zij met elkaar hebben op scholen en anderzijds door immunologische factoren, zoals een onrijpe adaptief immuunsysteem waardoor zij langdurig het virus blijven uitscheiden. [46-49] Vaccinatie van alle kinderen tegen influenza in plaats van alleen risicogroepen heeft ook voordelen voor niet-gevaccineerde personen via het fenomeen van groepsimmuniteit. [50-52] Wiskundige modellen op basis van gegevens van 14 influenza seizoenen in Engeland tonen aan dat vaccinatie van alle kinderen tussen 5-16 jaar gunstig en kosteneffectief is. [53] Vooral de combinatie van leeftijdsgroepen (lage en middelbare school) blijkt in deze statistische analyse kosteneffectief. [54] Op dit moment maakt seizoensgebonden griepvaccinatie met een quadrivalent nasaal en levend verzwakt griepvaccin (LAIV) al deel uit van het

RVP in Engeland. [42,43,55] De eerste berichten zijn bemoedigend. Er is zowel directe als indirecte bescherming opgetreden, wat resulteert in een verminderde incidentie van influenza-achtige ziekte en van zowel de bovenste en onderste luchtwegen. [56-58] De griepvaccinatie voor kinderen wordt geleidelijk uitgerold over meerdere delen van het Verenigd Koninkrijk.
Er is geen influenzavaccin beschikbaar voor kinderen die jonger dan 6 maanden zijn, hoewel de ziektelast juist het hoogst is bij kinderen in deze leeftijdsgroep. [45,59,60] Maternale vaccinatie zou in dit geval de ziektelast kunnen verminderen. Deze methode is veilig en wordt aanbevolen door de WHO sinds 2012. [61] Een recent gepubliceerde studie op de maternale vaccinatie in het seizoen 2013/2014 liet een effectiviteit van 71% qua aantal influenza infecties bij zuigelingen jonger dan 6 maanden en een effectiviteit van 64% ten aanzien van het aantal ziekenhuisopname. [62] Dit is in overeenstemming met een eerder gepubliceerde gerandomiseerde trial: 63% effectiviteit van het vaccin en 29% vermindering in periodes van ziekte van de luchtwegen met koorts. [63]

Conclusies en toekomstperspectieven van deel I van dit proefschrift

Kinderen met een risico op seizoensgriep lopen ook het risico op besmetting met ernstige influenza A H1N1-infecties. Een groot deel van de gehospitaliseerde kinderen met een ernstig beloop waren voorheen gezond. Het bewijs dat vaccinatie van kinderen zou leiden tot bescherming van de hele bevolking en ook kosteneffectief was, vonden wij in 2013 niet sterk genoeg. Echter, nieuwe gegevens over de druk van influenza bij zuigelingen en de medische sector, de kosteneffectiviteit van vaccinatie van gezonde kinderen en de voorlopige resultaten van influenza vaccinatie van kinderen in het Verenigd Koninkrijk suggereren nu dat Nederland opnieuw moet overwegen om het griepvaccin aan te bieden aan alle kinderen.
Alvorens dit te doen, zal het nodig zijn om de publieke perceptie met betrekking tot (griep)vaccinatie in Nederland te onderzoeken. Daarnaast is informatieverstrekking over de voor- en nadelen van het gebruik van dit vaccin bij kinderen noodzakelijk. Dit kan bezorgdheid over de veiligheid met betrekking tot griepvaccinatie, die een reden voor de weigering kan zijn, verminderen. [64-66] Nederland heeft momenteel een hoge respons bij griepvaccinatie bij risicogroepen (81%). [67,68] Echter, na de invoering van de HPV-vaccinatie bij jonge meisjes ontstond er veel discussie in de sociale media, de politiek en de wetenschappelijke gemeenschap ten aanzien van de veiligheid, de duur van de bescherming en de leeftijd waarop het vaccin werd gegeven. Dit illustreert het belang van informatie en voorlichting voor en tijdens de introductie van nieuwe vaccins.[69,70] Daarnaast is de logistiek van een uitgebreid influenzavaccin programma uitdagend, omdat er jaarlijkse een nieuw vaccin moet worden gegeven dat bescherming biedt tegen de voorspelde circulerende influenzastam. Studies uit het Verenigd Koninkrijk en de Verenigde Staten laten zien dat de school geïnitieerde vaccinatie het meest succesvol is en resulteert in een hoger percentage gevaccineerde mensen in vergelijking met vaccinatie door huisartsen en gezondheidscentra. [56,57] Daarnaast is maternale vaccinatie een effectieve manier om de ziektelast bij kinderen jonger dan 6

maanden te verminderen. Maternale vaccinatie wordt al geadviseerd in verschillende Europese landen. [71,72] Studies zijn nodig om te beoordelen of de jaarlijkse griepvaccinatie van gezonde kinderen ook de ziektelast bij kinderen onder de 6 maanden vermindert. Als dit aangetoond is, raden wij aan om een hogere prioriteit te geven aan de invoering van maternale vaccins tegen *Bordetella pertussis* en RSV omdat daar nog geen goed alternatief voor is.

Respiratoir syncytieel virus (RSV)

RSV werd zestig jaar geleden ontdekt. Infecties door dit virus worden wereldwijd beschouwd als een zware last voor kinderen en de maatschappij. [73,74] Tot nu toe is er geen behandeling beschikbaar en vaccins tegen RSV zijn nog in ontwikkeling. Toediening van palivizumab, een profylactisch monoklonaal antilichaam, biedt passieve bescherming voor hoog risico groepen, maar het is niet beschikbaar voor alle kinderen vanwege de hoge kosten. Bovendien werkt palivizumab toediening niet bij patiënten die al zijn besmet met RSV en veranderd ook het ziektebeloop niet als het gegeven wordt aan kinderen met een ernstige RSV-infectie. [75,76] Tot op heden is de behandeling van RSV infecties beperkt tot ondersteunende zorg, bestaande uit ademhalingsondersteuning en adequate vocht- en voedingstoestand.

De pathofysiologie van RSV infecties is complex en nog niet volledig bekend. Directe cytopathische effecten van het virus en versterkte ontstekingsreacties spelen een belangrijke rol bij de pathogenese van de ziekte. [77] Opname is bij RSV bronchiolitis noodzakelijk bij ongeveer 1-3% van zuigelingen en kinderen. [78] De ernst van de ziekte kan variëren van een milde verkoudheid tot respiratoir falen met de noodzaak tot kunstmatige beademing. De onderliggende oorzaak voor deze verschillen in ernst van ziekte is nog niet opgehelderd. [79-81] Risicofactoren voor een ernstig verloop van de ziekte, zoals prematuriteit, immuundeficiënties en aangeboren hart- en longaandoeningen zijn goed beschreven. [82] Meer dan de helft van de jonge kinderen op de intensive care was voorheen gezond. [83] Artsen gebruiken klinische parameters en de bekende risicofactoren om de ernst van de ziekte in te schatten en te oordelen of opname nodig is. Deze beoordeling heeft echter beperkingen aangezien ongeveer een derde van de gehospitaliseerde patiënten achteraf geen ondersteunende behandeling nodig had, terwijl 4,6-6,8% van de kinderen die vanaf de eerste hulp naar huis mochten later alsnog opgenomen moesten worden. [84-86]

Zoektocht naar biomarkers

In de **hoofdstukken 6-10** beschrijven we biomarkers die onderscheid kunnen maken tussen de groepen van ernst van de ziekte bij kinderen met virale lage luchtweginfecties (LLWI's). In de **hoofdstukken 6-8** bestudeerden we of de biomarkers die zijn beschreven in de context van (chronische) longziekte en bacteriële luchtweginfecties ook kunnen worden gebruikt om bij virale LLWI ernst van de ziekte te bepalen. In **hoofdstuk 6** toonden we aan dat een combinatie van inflammatoire eiwitten PTX3, CRP, SAA en Properdin patiënten met een ernstig ziekteverloop kunnen onderscheiden

van kinderen met een mild ziektebeloop van virale ziekte. De oppervlakte onder de curve (AUC) van deze combinatie van eiwitniveaus was 0,89. [87,88] In een validatie cohort van kinderen met virale LLWI had de test een sensitiviteit en specificiteit van respectievelijk 71% en 87%. Deze studie toont aan dat de strategie waarbij meerdere ontstekingsfactoren gecombineerd worden een aantrekkelijke benadering is voor het identificeren van biomarkers die ernst van ziekte voorspellen. Meting van PTX3, CRP, SAA en Properdin in een eerder stadium van de ziekte moet hun werkelijke voorspellende waarde aan het licht brengen. Bovendien is het interessant om te kijken hoe deze markers functioneren als voorspellers bij andere pediatrische infectieziekten.

In **hoofdstuk 7** bestudeerden we de expressie van oppervlaktemarkers op circulerende monocyten tijdens RSV-infectie en correleerden we de uitkomsten met ernst van ziekte. Monocyten zijn belangrijke aangeboren immuuncellen voor antivirale immuniteit. [89-91] Circulerende monocyten kunnen worden onderverdeeld in drie groepen (klassiek, intermediate- en niet-klassiek) op basis van hun CD14 en CD16 expressie. [92] We hebben aangetoond dat er significante verschuivingen in monocyten subpopulaties opgetreden tijdens acute RSV infecties. Veranderingen omvatten de expressie van oppervlakte-eiwitten CD14 en CD16, een verhoogde expressie van HLA-ABC en verminderde expressie van HLA-DR. Verminderde expressie van HLA-DR op monocyten correleert met de ernst van de ziekte. Dit verschijnsel is eerder waargenomen tijdens bacteriële infecties. [93-95] Of dit van prognostische waarde is, zoals werd aangetoond voor septische pasgeborenen is nog niet duidelijk.[93] Daarnaast werden *in-vitro* cytokineresponsen op endotoxine (LPS) gemeten bij gezonde controles en RSV geïnfecteerde patiënten met een ernstig en niet ernstig ziekteverloop. Patiënten met een ernstige RSV-infectie hadden normale TNF productie, maar vertoonde een lagere IL-10 productie vergeleken met de respons van gezonde controles. Dit komt niet overeen met 'immuun paralyse', een verschijnsel wat wordt gezien bij ernstige bacteriële infecties. Immuun paralyse wordt gekenmerkt door lage HLA-DR expressie en verlaagde TNF reacties. [93-95] Ernstige RSV infecties geven dus geen klassieke immuun paralyse, hoewel een onevenwichtige immuunreactie een belangrijke rol bij ernstige ziekte kan spelen.

Verhoogde serum concentraties van MMP8 en MMP9 zijn tijdens acute longbeschadiging en longontsteking bij volwassenen en kinderen aanwezig. [96-98] MMP8 en MMP9 eiwitten kunnen de extracellulaire matrix degraderen, helpen celmigratie van ontstekingscellen en hebben immunomodulerende capaciteiten. [99.100] MMP 9 niveaus zijn gecorreleerd met de ernst van astma en longontsteking. [96,97] In **hoofdstuk 8** hebben we MMP8 en MMP 9 eiwitniveaus en genexpressie gemeten in plasma en nasofaryngeale aspiraten om te zien of ze als een biomarker voor de ernst van RSV-ziekte kunnen dienen. [100] We zagen enkele verschillen in plasmaspiegels van MMP8 en MMP9 tussen kinderen met een milde en ernstige ziekte. De klinische waarde van MMP8 en MMP9 metingen als biomarker voor ernst van RSV ziekte is beperkt door een brede overlap tussen de groepen. Daarentegen heeft de meting van genexpressie niveaus van MMP 8 en MMP9 in zowel PBMC en granulocyten meer potentie om te worden gebruikt als een biomarker. Ook in een andere studie werd de potentie van MMP9 als biomarker voor de ernst van

RSV-infectie bij kinderen aangetoond. [101] *In vitro* studies met humane epitheelcellen tonen aan dat RSV een krachtige stimulans is voor MMP9 genexpressie en vrijlating. Bovendien kan MMP9 RSV infectie van humane epitheelcellen voorkomen *in vitro*, maar ook bij muizen longen. [101-103] In MMP9 knock-out muizen is de neutrofielen werving en cytokine productie aanzienlijk versterkt bij RSV-infectie. [103] Kong *et al.* tonen dat MMP9 knock-out muizen of muizen behandeld met siRNA om MMP9 genen onderdrukken minder inflammatie en verminderde neutrofiel influx hadden tijdens RSV-infectie. Ook was er verminderde virale replicatie van RSV. [102] Zij stellen dat overtollige MMP9 activiteit een nieuw doelwit voor therapieën kan zijn. [102]

Microarray studies

In **hoofdstuk 9** voerden we microarray analyse op PBMC's van kinderen met een primaire RSV-infectie uit. Door gebruik te maken van de *Prediction Analysis of Microarray* (PAM) analyse, werd Olfactomedin 4 geïdentificeerd als marker die patiënten met een ernstige ziekte herkent. Deze bevinding werd bevestigd in een cohort van kinderen met virale LLWI (zowel RSV positieve als negatieve kinderen). Het relatieve risico op de ontwikkeling van ernstige ziekte was, na correctie voor leeftijd en prematuriteit, bij kinderen met een hoog OLMF4 niveau 6,1. Ook nu bleken plasmaspiegels van Olfactomedin 4 minder discriminerend dan genexpressie niveaus, hetgeen overeenkomt met de data in **hoofdstuk 8**. Dit geeft aan dat de analyse van genexpressie in perifeer bloed soms gevoeliger en meer geschikt is als biomarker om ernst van ziekte te bepalen dan de meting van eiwitconcentraties. Verder concluderen wij, op grond van gegevens van andere microarray studies bij kinderen met virale infecties, dat de Olfactomedin 4 reactie niet RSV-specifiek is. Olfactomedin 4 kan dus ook een marker voor ernst van de ziekte zijn bij infecties veroorzaakt door andere virussen en bacteriën. Tot dusver is de biologische functie van OLFM4 nog niet volledig opgehelderd. Studies zijn vooral gericht op de rol van OLFM4 bij kanker, maar het blijkt dat OLFM4 ook een rol in de aangeboren immuniteit speelt. Bij infecties met *H. pylori* kan OLFM4 de *NF-k* B pathway downreguleren. [104.105] Daarnaast vertonen OLFM4 deficiënte muizen verbeterde immuniteit tegen sepsis en infectie door *Escherichia coli* en *Staphylococcus aureus*. [106] Die studie toonde aan dat OLFM4 een belangrijke negatieve regulator is van de bacteriedodende werking van neutrofielen door het beïnvloeden van cathepsine C-activiteit. [106.107] OLFM4 is een eiwit dat wordt opgeslagen in specifieke granules van een subset van circulerende humane neutrofielen (20-25%), tot nu toe is er geen verschil in functionaliteit tussen OLFM4-positieve en negatieve neutrofielen aangetoond. [108.] Opvallend is dat OLFM4 ook wordt uitgeworpen in *neutrophil extracellular traps* (NET), maar het gevolg ervan is niet bekend. [108.109]

Genexpressiestudies op PBMC's vereist een cel isolatie stap, die tijdrovend is en toepassing in de klinische setting belemmert. Daarom hebben we de protocollen aangepast om genexpressie op volbloed te meten. Eerst onderzochten we of een gen signatuur in volbloed kan worden vastgesteld dat het verloop van RSV-infectie bij kinderen met hoge nauwkeurigheid voorspelt.

In **hoofdstuk 10** werd de genexpressie data van volbloed van kinderen met een virale LLWI gecorrigeerd voor leeftijd en geslacht (confounders voor ernst van de ziekte) en geanalyseerd met verschillende bioinformatische benaderingen, met inbegrip van *machine learning*. Zo wilden we de ontdekking van biomarkers optimaliseren**.** Hiermee werd een expressieprofiel van 84 genen geïdentificeerd waarmee de patiënten met een ernstige RSV-infectie goed te onderscheiden waren van kinderen met een minder ernstig beloop. Deze set van 84-genen werd gevalideerd in een onafhankelijke cohort (dat gebruik maakte van een ander (Illumina) microarray platform, wij gebruikten Affymetrix) en toonde een AUC van 0.856 voor het identificeren van patiënten met een ernstig beloop van ziekte. De exacte klinische waarde moet in toekomstige studies worden geëvalueerd. Transcriptionele profilering kan worden gebruikt om bacteriële infecties te onderscheiden van virale ziekte. De groep van O. Ramilo was de eersten die verschillen in de gastheerrespons van kinderen aantoonde ten tijde van virale en bacteriële infecties, er bleken ook verschillen in genexpressie tussen de verschillende virussen en bacteriën onderling. [110.111] De groep van M. Levin publiceerde onlangs een gevalideerde set van maar twee genen waarmee op basis van expressie onderscheid gemaakt kan worden tussen bacteriële en virale oorzaken van ziekte bij kinderen met koorts. [112] Deze twee transcript RNA signatuur *(FAM89A* en *IFI44L)* heeft een hoge sensitiviteit en specificiteit (≥90%). [112] Een soortgelijk onderzoek werd gelijktijdig gepubliceerd met als doelgroep kinderen onder de 60 dagen met koorts, waarbij een gen set van 10 genen (inclusief MMP9) werd gebruikt. In een validatie cohort konden kinderen met ernstige bacteriële infecties worden geïdentificeerd met een sensitiviteit en specificiteit van> 90%. [113]

Een andere methode om transcriptoom data te analyseren is door alle expressie data van een ziek persoon bij elkaar op te tellen en deze uitkomst te vergelijken met het gemiddelde getal van een groep gezonde personen. Dit geeft het verschil in afstand tot de gezonde situatie aan (*molecular distance to health)*. [111] In **hoofdstuk 11** laten we zien dat de *molecular distance to health* van kinderen met RSV correleert met de ernst van de ziekte, de duur van de ziekenhuisopname en de duur van extra zuurstof. Deze methode wordt ook gebruikt in andere studies en lijkt een goede marker te zijn om ernst van ziekte te 'kwantificeren' bij infectieziekten, zoals tuberculose en bij kinderen met RSV of *Staphylococcus aureus* infecties. [111.114.115] De analyse van *molecular distance to health* lijkt een veelbelovende conceptuele benadering van ernst van de ziekte in infectieziekten bestuderen bieden. Het is interessant om te onderzoeken of dit ook geldt voor andere niet-infectieuze ziekten.

Chaussabel *et al.* ontwikkelde een modulaire analysemethode voor microarray studies, met deze methode wordt gekeken naar co-expressie van genen in plaats van één gen tegelijk. Op deze manier is het mogelijk om gedrag van celtypen en processen tijdens infecties te bestuderen. [116] In **hoofdstuk 11** werd deze werkwijze aangepast aan een Affymatrix microarray platform en toegepast op volbloed transcriptoom profielen om het inzicht in de pathofysiologie van ernstige RSV-infectie te verbeteren. Er werden zes modules geïdentificeerd die significante verschillen in genexpressie tussen de ernst-

groepen. De module van de neutrofielen had de meeste evidente verschillen tussen de ernstgroepen, met statistisch significant hogere waarden bij kinderen met een ernstig beloop van ziekte in vergelijking met kinderen die een mild en matig beloop van de RSV infectie hadden. Genexpressie in de modules van cytotoxische T-cellen en NK-cellen waren laag tijdens ernstige RSV-infecties. Dit is meest waarschijnlijk een gevolg van het lagere aantal cellen van dit type in volbloed zoals eerder al door onze groep werd getoond. [117] De modules inflammatie, bloedplaatjes en erytrocyten werden opgereguleerd. Vergelijkbare resultaten werden waargenomen in een andere uitgebreidere studie die de immuunrespons tegen verschillende virussen vergeleek, maar zich ook richtte op de verschillen in immuunresponsen bij patiënten met in RSV ziekte ernst. [111]
De rol van neutrofielen bij virale infecties is intrigerend en niet goed begrepen. Bij zuigelingen met een ernstig beloop van RSV wordt een sterke instroom van neutrofielen in bronchoalveolaire spoelingen waargenomen. Bij autopsies van zuigelingen die overleden aan een RSV infectie wordt een opeenstapeling van neutrofielen in het longweefsel gezien en slijmpluggen in de luchtwegen. [118.119] Het lijkt dat neutrofielen een belangrijke rol spelen bij deze luchtwegobstructie. [76] Onlangs hebben diverse studies aangetoond dat neutrofielen antivirale capaciteiten hebben. [120.121] *Neutrophil extracellular traps* (NETs) kunnen door RSV uitgelokt worden en deze NETs verhinderen dat RSV nieuwe cellen infecteren. Dit is echter niet alleen maar voordelig, want een overmaat aan deze NETs kan bijdragen aan een ernstiger beloop van ziekte. [121] Iversen *et al.* toonden aan dat een innate antiviraal pathway al kan worden geactiveerd door het epitheel, waardoor neutrofielen al vóór de interferon respons gerekruteerd worden. [120] NETs blijken ook bij andere virale infecties van belang, zoals influenzavirus, en lijken een rol te spelen bij de beheersing en preventie van virale infectie. [122 123] Het is intrigerend dat de biomarkers MMP8, MMP9 en OLFM4 uit onze studies allen kunnen worden opgeslagen in de granulae van neutrofielen. [108.124]

Mucosale biomarkers

In de voorgaande hoofdstukken kozen we ervoor om de systemische effecten van een lokale virale infectie te bestuderen. Het is moeilijk is om materiaal te verkrijgen uit de lagere luchtwegen van jonge kinderen. Analyse van parameters in perifeer bloed bleek toch een weerspiegeling te geven van de ernst van de ziekte zoals is besproken in het voorgaande deel van dit proefschrift. Het is reëel om aan te nemen dat de lokale ontstekingsreactie essentieel is in de ontwikkeling van ernstige ziekte. Daarom wordt in dit deel gekeken naar de immuunrespons in de neus-keelholte (nasofarynx). Een nasofaryngeale aspiraat (NPA) is relatief gemakkelijk te verkrijgen en is mogelijk een bron van potentiële biomarkers.
In **hoofdstuk 12** laten we zien dat het mogelijk is om een microarray te doen op materiaal wat verkregen is met een NPA (neusspoeling) (bevat epitheel en immuuncellen). Uit de analyse bleek dat een aantal genen, zoals tspan 8, MUC13, MSP en CCL7 significant verschilden in genexpressie niveaus tussen patiënten met milde tot matige en ernstige ziekte. Deze resultaten werden bevestigd met qPCR in een onafhankelijk

cohort. Transcriptie analyse van NPA lijkt een veelbelovende methode om prognostische testen te combineren met diagnostische procedures. Deze technologie biedt de mogelijkheid om de clinicus naast een microbiologische diagnose (RSV infectie) ook meteen een inzicht te geven in de ernst van de ziekte (de prognose).

Mucosale immuunrespons

Virale infecties van de luchtwegen ontstaan altijd in aanwezigheid van koloniserende bacteriën (microbioom). Bacteriële co-infectie kan niet altijd uitgesloten worden bij patiënten met een ernstige virusziekte. [125] In onze studies naar virale LWI konden routinematig kweken op de intensive care maar zelden een bacteriële co-infectie aantonen. De meeste kinderen met virale LLWI die niet op de IC opgenomen worden krijgen geen antibiotica, wat aangeeft dat bij niet-beademde kinderen bacteriële co-infecties geen belangrijke rol lijkt te spelen. De invloed van het respiratoire microbioom op de antivirale immuunrespons is nog niet uitgebreid bestudeerd. Interacties tussen enterale virussen, antivirale immuniteit en het darmmicrobioom krijgen toenemende belangstelling. [126-128] In **hoofdstuk 13** bleek dat zowel de aanwezigheid als de dichtheid van *Streptococcus pneumoniae* en *Haemophilus influenzae* in de nasofarynx niet correleerden met de viral load, mate van ontsteking of ernst van de ziekte bij patiënten met RSV infecties. Echter, bij patiënten die gekoloniseerd waren met *Streptococcus pneumoniae,* was de bacteriële dichtheid gecorreleerd met een hogere RSV load, hogere MMP 9 niveaus en een verminderde ernst van de ziekte. Dit impliceert dat de aanwezigheid van bacteriën inderdaad de antivirale gastheerreactie kan beïnvloeden. Onlangs publiceerde De Steenhuijsen Piters *et al.* dat het nasofaryngeale microbioom interactie heeft met de immuunrespons tijdens RSV-infecties en op deze manier mogelijk ernst van de ziekte kan beïnvloeden. [129] Zij toonden aan dat een bovenste luchtweg microbioom dat veel *H. influenzae* of *S. pneumoniae* geassocieerd is met een toegenomen ernst van de ziekte gedefinieerd als noodzaak tot ziekenhuisopname. In het modulaire genexpressie analyse op perifeer bloed van patiënten met deze verrijkte microbiomen, waren de genexpressies in genen met betrekking tot TLR-signalering en neutrofielen werving meer uitgesproken. Dit bevestigt de hypothese dat kolonisatie geassocieerd is met modulatie van de immuunrespons tijdens RSV infecties. Dit fenomeen werd ook gezien tijdens influenzavirus infecties. Deze studie gaf aan dat de resultaten impliceren dat het microbioom de immuunrespons tijdens respiratoire virale infecties reguleert en tevens de activatie drempel voor (innate) immuunresponsen kalibreert.[128.130] Suarez-Arrabal *et al.* beschrijven dat kolonisatie met Gram-negatieve bacteriën *(Moraxella catarrhalis* en *H. influenzae)* resulteert in verhoogde aantallen mucosale en systemische inflammatoire cellen, hogere pro-inflammatoir cytokine niveaus en een trend naar toenemende ernst van ziekte. [131] Dit is in overeenstemming met de studie van De Steenhuijsen Piters *et al.,*maar in tegenstelling tot de in **hoofdstuk 13** beschreven resultaten. Dit kan te wijten zijn aan verschillen in onderzoekprotocollen en detectiemethoden. We gebruikten een 16S qPCR specifiek voor *Streptococcus pneumoniae*, terwijl in de andere studies 16s rRNA gen amplicon sequencing werd gebruikt, waarbij de verschillende soorten

streptokokken niet te onderscheiden zijn. Bovendien, ontsteking was in onze studie gedefinieerd als eiwitniveaus, terwijl De Steenhuijsen Piters *et al.,* genexpressie gebruikte. Zoals in **hoofdstuk 8 en 9** al aangetoond is correleren plasma eiwitniveaus en gen expressie niet altijd.
De respiratoire mucosa en het slijm in de luchtwegen bevatten antilichamen. In het algemeen wordt gedacht dat immunoglobulines gericht tegen RSV een beschermend effect hebben, zoals weergegeven door de vermindering van ernst van de ziekte door passieve immunisatie met palivizumab en een beschermend effect van veel maternale antilichamen tegen ernstige RSV infecties bij kinderen in epidemiologische studies.[132-135] De RSV vaccinatietrial uit de jaren zestig, waarbij vaccinatie tegen RSV resulteerde in een ernstiger beloop van ziekte en zelfs tot meer sterfte bij kinderen met een goede antilichaamrespons, illustreert echter dat de antilichamen titer niet altijd correleert met de mate van bescherming. [136] Hoewel het exacte mechanisme van «antilichaam versterkte ziekte" nog niet duidelijk is, lijken de titers van niet-neutraliserende antilichamen en de mate van de aviditeit van antilichamen een belangrijke rol te spelen bij RSV-ziekte.
In **hoofdstuk 14** laten we zien dat er geen correlatie is tussen de titers in het plasma en de mucosale titers van IgG bij kinderen met virale LLWI's. Titers van mucosaal IgG correleren beter met de virale load en inflammatoire respons op de mucosa dan de titer van plasma IgG. Deze bevinding kan van belang zijn voor de evaluatie van toekomstige vaccinatiestudies bij kinderen. Ook kan dit een argument voor intranasale vaccinatie bij kinderen zijn. In muizen is het al aangetoond dat de mucosale CD8+ T cel respons na nasale vaccinatie beter is dan na intraperitoneale vaccinatie. [137]

Conclusies en toekomst perspectieven van deel II van dit proefschrift

De klinische waarde van de biomarkers die wij geïdentificeerd hebben moet nog bepaald worden in prospectieve studies met seriële metingen. Bij voorkeur moet er al op een eerder tijdstip, zoals bij het eerste contact met de huisarts gemeten worden. Tot nu toe kon slechts een beperkt aantal analyses worden uitgevoerd per patiënt vanwege een beperkte hoeveelheid bloed die veilig kan worden afgenomen bij jonge kinderen. Door verbeterde technieken is de hoeveelheid bloed per test drastisch verminderd (we gebruikten 3 ml bloed voor een micro-array analyse, dit kan nu al met 1,0 ml). [138] Hierdoor kunnen er meerdere analyses gedaan worden in een minimale hoeveelheid bloed, waardoor de ethische en veiligheidsargumenten tegen de seriële bepalingen afnemen. Zodra onze resultaten gevalideerd zijn kan een klinische studie opgezet worden. Op dat moment is aan het grootste deel van de criteria voor een goede biomarker, zoals voorgesteld door Morrow en de Lemos, voldaan. [139] Deze criteria stellen dat een goede biomarker gemakkelijk te meten moet zijn en informatie toe moet voegen aan klinische besluitvorming. De marker moet dan ook een impact hebben op het klinische besluitvorming. [139] Sequentieel testen kan hierbij ook een toegevoegde waarde hebben, omdat de kinetiek in de tijd mogelijk een betere prognose geeft. Het gebruik van een combinatie van biomarkers zou de gevoeligheid en de specificiteit van deze tests ook kunnen verhogen, zoals beschreven in **hoofdstuk 6**. Zodra een ideale combinatie van

genen of eiwitpanel is vastgesteld, kan een point-of-care test ontwikkeld worden. De techniek voor bedside PCR en eiwitmeting in plasma is al beschikbaar. [140.141]
Toekomstige studies moeten uitwijzen of het aantal genen om de *molecular distance of health* te meten ook gereduceerd kan worden tot een beperkt aantal genen, dit zou het gebruik van deze maat in de kliniek reëler maken. De modulaire analyse van Chaussabel *et al.* kan niet alleen worden gebruikt om meer inzicht te krijgen in de pathofysiologie van ernstige RSV-infecties, maar kan ook worden toegepast op andere (infectie) ziekten. De gecombineerde analyse van genomische data, microbioom analyse en RNA sequencing, kan het inzicht omtrent de wisselwerking tussen het microbioom en de humane immuunrespons verhogen, vooral bij gebruik in een experimenteel *humaan challenge model*. Integratie met andere 'omic' technieken, zoals metabolomics en proteomics, zal een grondige analyse van de gastheer-pathogeen interactie vergemakkelijken en biedt de mogelijkheid om individuele gevoeligheid ontcijferen voor ernstige RSV-infecties. [142]
Op dit moment is alleen ribavirine goedgekeurd voor de behandeling van RSV. [143] Het gebruik van ribavirine bij ernstige RSV-infecties is controversieel en wordt niet aanbevolen. Het is moeilijk om ribavirine te dienen in beademde patiënten op de intensive care en het geneesmiddel is duur. [143.144] Verschillende antivirale middelen worden momenteel onderzocht. Potentiële nieuwe antivirale middelen kunnen worden onderverdeeld in drie groepen op basis van hun werkingsmechanisme, 1) nucleocapside eiwit remmers (RSV604), 2) RNA-afhankelijk RNA polymerase inhibitoren (YM-52403, BI-DD en ALS-8176) en 3.) fusie-remmers (TMC-353121, BMS-433771, VP-14637 en GS-5805). [145-149] Momenteel worden alleen ALS-8176 en GS-5806 getest in klinische studies. [150.151]
Door de ontrafeling van de kristalstructuur van het F-glycoproteïne van RSV in zijn pre-geactiveerd (prefusion) conformatie heeft de vaccinontwikkeling een boost gekregen. [152.153] Neutraliserende antilichamen binden hoofdzakelijk aan de prefusion vorm van het F-eiwit (PREF). [154.155] Dit PreF is zeer instabiel en eenvoudig schakelaars zijn postfusion vorm (postF), die geen functie in de fusie van membranen en virale intrede heeft in een gastheercel. [156.157] Daarom bieden antilichamen gericht tegen de postF vorm enkel beperkte bescherming. De ontwikkeling van monoklonale antilichamen specifiek gericht tegen de preF is zeer effectief in het verminderen van virale infectie. [155.158] Zodra de halfwaardetijd van deze antilichamen is verhoogd kan dit worden gebruikt voor passieve immunisatie. Dan is slechts één toediening nodig om ernst van RSV-ziekte te verminderen in de eerste 6 maanden van het leven.
Intranasaal toegediend levend-verzwakte vaccins of chimerische levende vector vaccins worden momenteel getest in klinische trials en lijken veilig te zijn voor gebruik bij kinderen ouder dan 6 maanden. [159-162] Verder worden gen-gebaseerde replicatie-defecte vaccins met virale vectoren of nucleïnezuren als veilig beschouwd. De vaccines zitten nu in de vroege fase van klinische onderzoek bij volwassenen. [159.160] Subunit eiwit en eiwitten op basis van nanodeeltjes zullen waarschijnlijk voor maternale vaccinatie doeleinden gebruikt gaan worden om de jongste kinderen te beschermen. [160] Andere vaccinatiestrategieën zoals virus-like partikels en virosomen zijn inmiddels getest in dierstudies, maar nog niet toegepast in klinische studies. [159]

De huidige klinische trials met antivirale middelen en vaccins bieden de mogelijkheid voor toekomstige generaties om de epidemiologie van RSV ziekte te veranderen. De beschikbaarheid van gevalideerde biomarkers om ernst van virale ziekte te voorspellen kan ook van nut zijn voor het ontwerp van toekomstige studieprotocollen bij onderzoek naar de werkzaamheid van antivirale middelen en vaccins. Ook op deze manier kan een biomarker een bijdrage leveren aan het verminderen van een ernstig beloop van een RSV infectie.

Literatuur

Referenties staan bij hoofdstuk 15.

Curriculum Vitae

Inge Maria Leonie Ahout werd op 17 februari 1984 geboren in Apeldoorn. Toen ze zes jaar was verhuisde ze naar Vaassen. Zij behaalde haar VWO diploma aan het Stedelijk Gymnasium in Apeldoorn, zij volgde het profiel Natuur en Gezondheid met de extra vakken natuurkunde-2 en scheikunde-2. Van 2002 tot 2008 studeerde zij geneeskunde aan de Universiteit van Maastricht. Daarna heeft zij gedurende ruim 1 jaar als arts-assistent niet in opleiding gewerkt in het Canisius Wilhelmina Ziekenhuis te Nijmegen. In februari 2010 begon zijn aan haar promotietraject “Determinants of disease severity in children with viral lower respiratory tract infections” onder de begeleiding van dr. J.G. Ferwerda, dr. M.I. de Jonge, Prof. dr. R. de Groot en Prof. dr. P.W.M. Hermans binnen het Laboratorium Kinderinfectieziekten van het Radboudumc. Zij participeerde in het VIRGO-consortium, hierbinnen was ze verantwoordelijk voor het includeren van patiënten met acute virale luchtweginfecties op de eerste hulp, polikliniek en de kinderafdeling van het CWZ en het Amalia kinderziekenhuis, en van de intensive care afdelingen van het Radboudumc Nijmegen en Sophia kinderziekenhuis Rotterdam. Ook werden hiervoor door bijna heel het land huisbezoeken afgelegd. Voor de studie naar de griep epidemie met H1N1 in 2009 werden (bijna) alle ziekenhuizen met een kinderafdeling bezocht. Hiervoor werkte ze intensief samen met Mariëtte Las (RSV) en Ria Philipsen (H1N1) die nauw betrokken waren met de logistiek en inclusie van de patiënten. Ook begeleidde ze diverse studenten geneeskunde tijdens hun wetenschappelijke stage. De resultaten van haar onderzoek zijn grotendeels beschreven in dit proefschrift. In mei 2014 startte zij haar opleiding tot kinderarts met een perifere stage in het Canisius Wilhelmina Ziekenhuis in Nijmegen (opleider dr. B.A. Semmekrot). Momenteel vervolgd ze haar opleiding binnen het Amalia kinderziekenhuis Radboudumc te Nijmegen (opleider dr. J.M.T Draaisma en later dr. A.A.E.M. van Alfen - van der Velzen).

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